



GENE THERAPY TECHNOLOGIES, APPLICATIONS AND REGULATIONS

From
Laboratory
to Clinic

EDITED BY
ANTHONY MEAGER



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Gene Therapy Technologies, Applications and Regulations

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Preface

There have been many new beginnings in the twentieth century regarding the treatment, prevention and diagnosis of human diseases and medical conditions. The latest, 'gene therapy', perhaps offers the greatest potential for radical new therapies. Gene therapy, as currently perceived, encompasses a set of procedures deliberately aimed at the efficient transfer of genetic material into the somatic cells of individual patients in order that the expression, or other function, of the part(s) of the genetic material designed to elicit preventive, therapeutic or diagnostic activity is fully functional and effective for its intended purpose. Originally, gene therapy was conceived as a way of correcting genetic defects in human inherited genetic disorders, of which there are now more than 4000 known. For most of these genetic disorders, there are no satisfactory treatments.

With increasing knowledge of human genes, gained largely through the Human Genome Project, it will eventually become possible to identify all of the genes and the mutations therein which underly genetic disorders. It should then be possible to contemplate replacing or compensating deleterious alleles with normal ones in those somatic cell populations most closely associated with disease manifestations. The precise correction of genetic defects is the ultimate goal of investigators developing gene therapies. The ideal strategy in inherited monogenic diseases would be to exchange a defective gene, or its damaged parts, for a normal gene by homologous recombination. However, while homologous recombination has been generally viewed as the best approach for gene correction, it is of far too low efficiency at present to be of any use. Moreover, current vector-mediated transgene delivery systems are unable to target specific sites within the human genome. They either deliver the transgene to cytoplasmic or episomal sites, where it remains unintegrated and is transiently expressed, or to random integration sites within nuclear chromosomal DNA, where it is expressed in unpredictable amounts. Therefore, at best, current gene therapies result in added normal (trans-) gene expression, which may fully or partly compensate genetic defects. It may be conceived that in somatic gene therapy of this kind the normal gene is used as a drug in the sense that it acts as the means for producing a gene product, a protein, *in situ*. The approach may be considered as only slightly different from using gene products themselves, e.g. insulin, factor VIII. However, somatic cell gene

therapy has not only the potential to ameliorate symptoms, but also appears to offer the prospect of a 'genetic cure' in sufferers of genetic disorders.

It has been quickly realised that there are wider applications of somatic gene therapy beyond treatments of genetic disorders that potentially could evolve as beneficial treatments for a whole range of human multifactorial diseases. These include cancers (malignancies), arthritis, atherosclerosis, neuropathies, autoimmunity and infectious diseases, such as human immunodeficiency virus (HIV) infections. In fact, the potential number of recipients of gene therapy in the latter category of diseases far outweighs that for the comparatively rare monogenic disorder category. Furthermore, in diseases such as cancer, there are several different metabolic and immunological targets that create a number of opportunities to design distinct and potentially efficacious gene-mediated therapies. For example, cancer cells can be induced to undergo cell suicide (apoptosis) by introduction of a normal gene, e.g. p53, to compensate a mutated one; they can be killed by conversion of a pro-drug into a toxic metabolite if the gene encoding the relevant converting enzyme is transferred to them, e.g. herpes simplex virus thymidine kinase with ganciclovir; they can be destroyed by leukocytes activated by (i) the expression of cytokine transgenes, e.g. interleukin-2, tumour necrosis factor alpha, or (ii) the expression of cell surface molecule transgenes which increase immune recognition, etc. In other diseases, in particular in HIV infections and AIDS, where there are a variety of symptomatic complications, a similar diversity of gene therapy strategies can be expected.

It should be emphasised that somatic cell gene therapy is still very much in its infancy as a treatment modality. First attempts to use gene therapy, for example for the treatment of β -thalassaemia in a clinical trial in 1980, were misguided, technologically unsound and not even ethically approved. It was another ten years before a sound, approved protocol was developed. At 12.52 p.m. on 14 September 1990, Dr Kenneth W. Culver started the infusion of genetically modified autologous lymphocytes into a four-year-old girl with adenosine deaminase (ADA) deficiency. This event, the true 'birth' of gene therapy, started the bandwagon rolling and it has been gathering speed ever since. In the short space of eight years or so there has been an exponential research effort to design and produce vectors to transfer genetic material to somatic cells and an unprecedented rush into clinical trials. It has to be said that gene therapy suffered media 'hype' and expectations were raised too high. Nevertheless, preliminary clinical studies, which have largely been phase I clinical trials to determine safety and feasibility, not efficacy, have looked promising. Furthermore, the obstacles to success, which appear formidable, are now appreciated. Scientific advances, technological improvements in vector design, and further under-

standing of pathophysiological mechanisms will be required to overcome the barriers that currently impede the successful application of gene therapy.

There are many concerns about the safe application of gene therapy. In this case, it is the nature of the therapeutic agent, i.e. potentially heritable DNA, that has raised attention and public concerns. Obviously, we, during our natural lives, endure endless assaults by potentially pathogenic and invasive microorganisms that 'load' us with their genetic material; most of it seems to be 'resisted', but some gets integrated, e.g. from retroviruses and lentiviruses. This is 'accidental', not deliberate as in gene therapy. Thus, ethical and moralistic issues are raised principally because gene therapy appears to be 'human genetic engineering'. Gene therapy involves the deliberate transfer of potentially heritable genetic material into human cells. At present, most nations have considered any intentional transfer of genetic material into germline cells to be unethical, even morally repugnant, since the 'added genes' could be transmitted to offspring, with unforeseen consequences. Therefore, the only gene therapy that has been permitted is that applied to human somatic cells, cells that are incapable of transmitting genetic material to offspring. Nevertheless, there remain several proponents for the use of germline gene therapy; arguments have been raised to the effect that it should not be completely banned as there may come a time when technological advances enable it to cure, and possibly eradicate, otherwise intractable genetic diseases. It is likely, however, that even if circumstances arose that warranted such applications, there would still be considerable opposition from those who consider the use of such technology morally wrong. One difficulty that would probably arise is how medical conditions and traits (natural variations) could be absolutely distinguished. How would baldness, for example, be considered? If germline gene therapy could prevent this late-developing condition/trait, then there might be pressure from 'sufferers' to try to avoid baldness in their offspring and future generations in their families. Would parents given the opportunity to produce 'brainier' children resist the temptation if germline gene therapy could offer this advantage? It is clear that in the future some difficult decisions will have to be made as technology progresses to the point where manipulation of the germline becomes a real feasibility.

If there are dangers and public conflicts about using germline gene therapy in the future, there are also certainly many aspects of somatic cell gene therapy as now practised that have come under the spotlight. These require our best scientific knowledge and regulatory frameworks to ensure the safe application of somatic cell gene therapies and continued public acceptance of these novel technologies and treatment modalities.

The feasibility of human somatic cell gene therapies has been enabled by pre-existing and developing technologies for preparing modified nucleic

acids and the means for transferring genetic material into cells. Currently, a number of possible ways of constructing delivery vehicles, otherwise known as vectors, are being investigated, with the ultimate goal of attaining efficient transfer of genetic material to intended target cell populations and, contingently, the appropriate level of transgene expression for resolution of the clinical condition being treated. In general, to form vectors, transgenes may either be linked into suitable plasmids and complexed with a variety of inorganic and organic chemical matrices, or be incorporated in the genomes of a number of different viruses, e.g. retroviruses, adenoviruses, adeno-associated viruses and herpes simplex viruses. Viral vectors have been, and continue to be, the primary choice of many investigators because they offer more efficient packaging and transfer of transgenes than complexed DNA, e.g. with cationic liposomes. However, despite the removal of certain viral genes to ensure the resulting viral vectors are replication-deficient except in specialised 'packaging' cell lines, there remain safety issues regarding their use. In addition, there remains much to be learnt with regard to targeting viral vectors to their intended target cell population and to the regulation of transgene expression once inside their target cell. Vectors may be used to transfer genes directly *in vivo* or to genetically modify cells in culture by an indirect *ex vivo* method. Both approaches have advantages and disadvantages. *Ex vivo* gene transfers with somatic cells that can be removed from the body and then put back once the cells are genetically altered and express the transgene product are more efficient and more readily controlled than *in vivo* methods. However, relatively few human tissues, e.g. haematopoietic, skin, endothelial and tumour cells, are amenable to the *ex vivo* gene transfer approach, since most tissue cells, e.g. from kidney, liver and brain, do not survive or grow well in culture. An alternative strategy of encapsulating genetically altered, allogeneic (or xenogeneic), cells, e.g. fibroblasts in immunoprotective polymers, and implanting these in relevant anatomical sites where they continuously secrete the transgene product without the danger of immunological rejection, may be, with further technological advances, a way of circumventing this limitation.

Potentially, there is a very wide applicability of somatic gene therapy to human diseases. Furthermore, in many diseases, a variety of gene-mediated therapeutic strategies appear of possible benefit. Therefore, there has been and is great interest from an increasing number of pharmaceutical companies seeking to explore and eventually exploit gene therapy technologies and products. Many of these technologies have originated on a small scale in R&D laboratories, but, as in the case of development and application of all other biological medicines, facilitation of technology transfer from laboratory to clinic requires appropriate quality controls and safety testing on gene therapy products (vectors and genetically modified cells), and procedures (protocols) are applied to (i) minimise patient risk, (ii) protect public health,

and (iii) maintain public confidence. As gene therapy technologies move towards major industrial commercialisation, discussions among representatives of regulatory bodies, health authorities, the medical profession, the scientific establishment and the pharmaceutical industry have taken place, and are still in progress, to develop regulatory frameworks that ensure the quality, safety, efficacy and ethical acceptability of products (vectors) and procedures (protocols). Such regulatory frameworks have to take account of the amorphous nature of gene therapy technologies and their nebulous applications and thus are decidedly complex. The aim of the regulators is, however, to ensure that products and procedures are safe and ethical to use without, at the same time, impeding the development of this emerging field of gene-mediated therapies.

This book sets out firstly to review many of the current technologies for preparing vectors for use in gene therapy protocols. Secondly, a range of medical conditions, including both inherited disorders and acquired diseases, that could potentially benefit from the application of gene-mediated therapies in somatic cells is well reviewed. The therapeutic potential of gene therapy protocols is, in many cases, compared with existing, alternative treatments. (Regrettably, cancer gene therapy could not be covered due to space limitations). Thirdly, regulatory themes are considered, including product quality and safety requirements. Finally, the transfer of technologies from laboratory to clinic is appraised with regard to the attendant requirements and facilities for (i) good laboratory practice (GLP) conditions in the R&D laboratory, (ii) large-scale production methods and good manufacturing practice (GMP) and (iii) current in-process and final product testing. The Chapters in this area provide information to those embarking on gene therapy technologies relevant to specifications of production and testing of products (and procedures) required to meet existing regulations, including quality, efficacy and safety considerations.

A panel of experts from the major industrialised countries, dealing with many aspects of human somatic gene therapy, have contributed to this book to ensure that a balanced view and global picture of the technologies, applications and regulatory requirements is presented. It is my hope that this volume will lead to the dissemination of the accumulated knowledge contained within and to the advancement of technologies and procedures pertinent to gene therapy.

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1 Cationic Liposomes for Gene Therapy Applications

J.P. CLANCY and ERIC S. SORSCHER

1.1 INTRODUCTION

Cationic liposomes are synthetic vectors that mediate the delivery and expression of transgenes within mammalian cells. Considerable progress has been made in several areas towards the development of these vectors. The mechanisms by which the reagents act has become better understood, and improved lipids for specific gene transfer applications *in vivo* have been identified. Moreover, the flexibility inherent in the strategy has been exploited towards novel approaches to improving the technology and to treating human diseases. Lipid–DNA complexes have been applied to preclinical and clinical trials, with encouraging preliminary results in a variety of human models.

1.2 MECHANISM OF ACTION OF CATIONIC LIPOSOMES

Felgner *et al.* (1) first described the use of lipid molecules with a positively charged head group for gene transfection into cultured cells. The efficiency of gene transfer was facilitated by inclusion of a neutral lipid molecule, such as dioleoyl phosphatidylethanolamine (DOPE) in the formulation. The method is comparable in efficiency to most other non-viral approaches to gene transfer *in vitro* and has been effective in most cell types studied. The mechanism underlying the process, and the features that limit lipid-mediated gene transfer, are not completely understood. Unlike other lipid-based drug delivery systems, plasmid DNA is not encapsulated within the core of a spherical liposome, but instead the DNA becomes heavily condensed by cationic lipids that completely or partially cover the plasmid. This condensed and coated form of the DNA can be characterized by electron microscopy (2) or by measuring the refractoriness of DNA to nuclease treatment *in vitro*.

Although it was initially suggested that lipid–DNA complexes fuse directly with the plasma membrane and subsequently enter the cytoplasm, it is now believed that in many cases the uptake of plasmid DNA requires endocytosis, and can be augmented either by endosomolytic drugs such as chloroquine or by the addition of viruses (e.g. adenovirus) capable of mediating endosomolysis (3–5). Efforts aimed at improving the effectiveness of lipid-mediated gene transfer *in vitro* have also focused on the development of new lipid reagents designed to augment fusion with the cell membrane. Efficacy of transfection of a particular cell type in culture often requires optimization of the lipid:DNA ratio or the proportion of cationic to neutral lipid. However, once an effective composition is found, in general it does not appear that delivery of plasmid to the cytoplasm is a major aspect that limits the overall process. Highly efficient delivery of fluorescent plasmid molecules or fluorescent nucleotides has been shown in several cell types, and delivery of DNA to nearly 100% of cells in culture has been substantiated both by electron microscopy and by techniques in which plasmid molecules are transcribed directly in the cytoplasm, indicating nearly 100% delivery of plasmid in many cases (6,7). Cytoplasm release of plasmid DNA from cationic liposomes is another important feature in the efficiency of the overall process (8).

The impressive effectiveness of plasmid DNA delivery to non-polarized, cultured cells contrasts with the relatively low percentage of cells that actually express detectable transgene by this method. Even under optimized conditions, only 1–5% of transfected cells in many cell types may express detectable levels of a β -galactosidase transgene, if judged by a relatively insensitive assay such as X-gal histochemistry. This discrepancy has led to the hypothesis that release of plasmid molecules from cationic lipids and entry of plasmids into the nucleus represent substantial impediments to gene expression with cationic liposomes (6). It has also been suggested that cell division, and concomitant dissolution of the nuclear membrane, may be a requirement for plasmid-based gene transfer, and that in tissue culture only the small proportion of cells undergoing active mitosis at the time of plasmid delivery is capable of conveying cytoplasmically situated plasmids to the nucleus for transcription. Attempts to target plasmid molecules through the nuclear pore by attaching nuclear localization signals have indicated a modest improvement in gene transfer efficiency in non-polarized cells *in vitro*. However, the role of the nuclear membrane in lipid-mediated gene transfer is likely to be complex, since prolonged exposure of cells to lipid–DNA complexes (to allow a larger proportion of cells to undergo cell division during the period of plasmid delivery to the cytoplasm) does not consistently augment the efficiency of gene transfer or expression. Moreover, attempts to transfect synchronized cells have not improved transfection efficiency. When very sensitive methods for detecting gene expression are used, most cells in a population appear both to receive and to express foreign genes (7). On the

other hand, certain cells within a population express markedly higher levels of transgene, but the reasons for this high-level expression have not yet been identified.

1.3 LIPID-MEDIATED GENE TRANSFER TO POLARIZED EPITHELIUM

The relatively poor ability of cationic liposomes to transfect differentiated epithelial cells suggests that the acquisition of polarity and tight junction formation may represent a special case that limits delivery of plasmid DNA molecules. *In vitro*, the growth of epithelial cells as a high-resistance monolayer on permeable supports is often used to model the *in vivo* situation in lung, intestine or other epithelial tissues. Under these conditions, uptake and delivery of DNA appears to be very limited, and a block of plasmid entry into the cytoplasm may substantially interfere with gene transfer efficiency. Permeation mediators such as sodium glycocholate may enhance gene transduction of murine lungs, but these agents also increase toxicity (9). At least one model has indicated a strong dependence of transfection on endocytosis in polarized, differentiated cells (10). Whether this block may be overcome at very high doses of lipid–DNA complex *in vivo* is not known. In addition, newer generation lipid formulations may have an improved ability to transfect epithelial monolayers. These results point to the importance of carefully selecting appropriate models in order to examine the efficiency of cationic lipids *in vitro*, or to predict biologic effectiveness *in vivo*. Differentiated epithelium (in tissues such as lung, gastrointestinal tract, or solid tumors) requires special considerations if it is to become a target for efficient lipid-mediated gene transfer *in vivo*.

1.4 CATIONIC LIPID DRUG DISCOVERY

The development of improved cationic lipids for gene transfer has depended on empiric testing of libraries of compounds, each of which is screened for a particular application. In general, it is advantageous to include a neutral lipid (generally DOPE) in the final formulation. First-generation compounds such as DOTMA (1,11) and DOTAP have not been supplanted by lipids containing polyamine head groups. Under specified conditions, newer reagents clearly lead to improved transfection efficiency. However, optimization of a particular formulation for each cell type and application must still be performed for each cationic liposome formulation.

For *in vivo* applications, large-scale screening of liposome vectors has successfully identified a number of very active compounds. DMRIE (1,2-

dimyristyloxypropyl-3-dimethylhydroxyethylammonium bromide) has been shown to effectively transfer reporter and therapeutic genes to established solid tumors in preclinical and clinical trials (see below). GAPDLRIE (12) also appears to substantially enhance potency during *in vivo* studies, for example in gene delivery and expression to intimal and medial cells within porcine arteries (13). Several lipids (including DOTMA and later generation compounds) formulated specifically for intravenous delivery elicit strong reporter gene signals in many tissues, including lung and liver (14,15). In some settings, liver uptake may be predominant within the Kupffer cells of the reticuloendothelial system. In mice, liposomes specifically formulated for gene transfer to the lung have been identified (16,17). GL-67, for example, given as an aerosolized complex with plasmid DNA leads to gene transfer in the lungs of mice approximately 1000-fold greater than plasmid DNA alone, and with potency approximately 100-fold greater than other, optimized lipids used in pulmonary gene transfer (16). Although this class of compound was identified by high throughput screening of large libraries of cationic liposomes, structure–activity analyses of the compounds have begun to emerge. Studies of similar reagents in the same class as GL-67 suggest that a T-shaped cationic head group comprised of a cholesterol anchor linked to spermine greatly augments gene transfer to murine lungs *in vivo*. Modification of critical regions in the GL-67 molecule should allow further improvements in activity using the methods of conventional medicinal chemistry. Guanidium-cholesterol cationic lipids are also reported to mediate efficient gene transfer to mammalian lungs (18). However, a particular lipid with activity in a particular tissue (e.g. GL-67 in lung) does not necessarily imply high potency when other tissue types or applications are considered. This indicates that the development of cationic liposomes for *in vivo* use may require considerable screening and empiric testing for a particular application.

1.5 LIPID RETARGETING

Failure of fusion of lipid–DNA complexes with target cells and inefficient plasmid entry into the cytoplasm have been suggested as important features that limit lipid-mediated gene transfer *in vivo*. Modifications of liposomal complexes by incorporating the Sendai virus fusion protein have been reported to improve both *in vitro* and *in vivo* gene transfer in several contexts, including recombinant insulin expression at levels suitable for lowering serum glucose in the mouse, delivery of antisense oligonucleotides in order to block neointimal proliferation, gene transfer to mammalian retina, and targeting of glioma tumors (19–21). Human angiotensin-converting enzyme (ACE) delivered by this technique into rat carotid arteries

led to ACE expression in both medial smooth muscle and intimal endothelial cells. Vascular proliferative changes were observed in the target arteries (22), and expression in a rabbit carotid arterial wall model has also been reported (23). The same approach also transfers the superoxide dismutase gene to a high percentage of cardiac myocytes in a rat heart transplantation model (24). Targeting of cationic lipids towards specific receptor-mediated endocytic pathways has also been reported. For example, in an attempt to obviate problems with cationic lipid inactivation in the circulation, DNA was complexed with lipopolyamine or other ligands. This design was intended to drive uptake through the asialoglycoprotein receptor, and *in vitro* evidence of uptake by this mechanism was demonstrated (25). Cationic lipids can be stabilized for *in vivo* gene delivery by incorporating polyamines or polyethylene glycol phospholipid molecules in the formulation. Likewise, attempts to incorporate cationic lipids into synthetic 'artificial viruses' (26) have been proposed for the purpose of non-viral vector targeting.

1.6 PLASMID MODIFICATION

Two major efficacy endpoints that may require substantial improvement in the context of lipid mediated gene transfer are (i) low levels of expression, and (ii) short duration of expression (27). Several strategies have been tested *in vitro* to address these issues. Nuclear targeting sequences covalently attached to the plasmid lead to small increases in reporter gene activity (28,29). Similarly, transactivation by T7 polymerase leads to increased gene expression *in vitro*, although the duration of plasmid persistence was not markedly augmented by this technique (30). Judicious selection of gene regulatory elements can lead to substantial increases in detectable gene transfer efficiency as mediated by cationic liposomes. For pulmonary gene transfer *in vivo*, modified cytomegalovirus- (CMV-) based promoters have been reported to confer levels of gene expression 100-fold higher than first-generation constructs in the same setting (12). The 5' flanking region of *c-erb* B-2 was used to specifically direct expression of the herpes simplex virus-thymidine kinase (HSV-tk) gene in pre-established tumors in mice (31). Plasmids based on Epstein-Barr virus sequences delivered to the liver by cationic liposomes persisted for several months, apparently by directing episomal, plasmid self-replication (32). Plasmid replicons capable of self-perpetuation in epithelial cells based on incorporation of the human papilloma virus (HPV) E1, E2, and the upstream regulatory region have also been reported (33). These HPV elements are strong transactivators, and lead to approximately 10 000-fold enhancement of reporter gene signals in epithelial cells in culture.

1.7 PRECLINICAL AND CLINICAL TRIALS

Several human studies using cationic liposomes have reported encouraging results after nasal installation with cystic fibrosis transmembrane conductance regulator (CFTR) plasmid DNA (34). Trials to establish (i) lower airway administration of aerosolized lipid–DNA complexes, and (ii) re-administration of lipid–DNA to the nasal airways are also now complete. In general, these studies have indicated some measure of gene transfer, with minimal side effects in nasal protocols, and some systemic toxicity in the lower airway trials. Advances in liposome-based technology have also led to the development of preclinical tests for many other human gene therapy applications. Gastrointestinal delivery and expression of the wild-type adenomatous polyposis coli (APC) gene (35,36), delivery of α -1 antitrypsin to the liver (37), delivery of factor IX (38) to mouse liver and other organs, reporter gene expression in hematopoietic stem cells (39), and intact vertebrate embryos *in vivo* (40) have each been performed successfully in animal models using cationic liposomes. Gene transfer of the α -1 antitrypsin gene to liver could be substantially prolonged (> 5 months) if performed in association with a partial hepatectomy (41). Liposome-mediated gene transfer (*ex vivo*) into murine bone marrow cells led to detectable gene activity four weeks following reinfusion of the transfected cells (42). Cationic liposomes have also been used to inhibit intimal smooth muscle proliferation (such as by delivery of E2F binding site decoys) for *in vivo* studies of rat carotid injury (43,44) or to deliver genes encoding interleukin-10 (IL-10) or domains of the human tumor necrosis factor alpha (TNF α) receptor to mitigate lethal endotoxemia in mice (45). Liposome-mediated expression of the luciferase gene in pig coronary arteries could be augmented further by activation of vascular cell proliferation. In a rabbit aorta model, DOTMA/DOPE was used to transfer human growth hormone cDNA, although only a small fraction of arterial cells ($< 1\%$) expressed the gene product *in vivo* (46,47).

Ocular tissues including cells of the cornea, iris, ciliary body, and retina have been transfected *in vitro* and *in vivo* using cationic lipids as part of the development of new approaches to diseases of the eye (48,49). Clinical development of strategies such as these for human gene transfer is in progress.

Following initial studies, by Canonico *et al.* (50), preclinical gene transfer to mammalian airways has now been performed successfully by several approaches. These studies include installation of both early generation lipids (DOTMA, DOTAP, DMRIE, DC-cholesterol) and later generation reagents such as GL-67 (see above). Correction of airway bioelectric defects in the cystic fibrosis mouse model (51–53) have been shown using lipid-mediated gene transfer. Intravenous administration of lipid–DNA complexes, for example using DOTMA/DOPE, leads to gene expression in surface airway epithelial cells *in vivo*. Gene expression signals in some cases have been

reported to persist for several months by this technique (54). Cationic or other lipids may lead to complement activation in the blood *in vivo*, but complex formation with DNA appears to reduce this tendency (55). Radiolabeled plasmid delivery can be tracked in animal models by whole body autoradiography (56)

Cationic liposomes have been used to deliver therapeutic genes to established tumors, or as tumor vaccines in an adjuvant setting. Small amounts of HLA-B7 (micrograms of DNA delivered to tumor tissues) complexed with DMRIE/DOPE or DC-cholesterol have been safely inoculated into melanoma tumors in preclinical studies and in human subjects. This strategy is designed to illicit a strong immunologic response against tumor antigens. Objective tumor regressions have been observed in some patients (57,58), and anti-tumor effects at a distance (i.e. in melanoma nodules not directly uninoculated with lipid-DNA complexes) have also been noted. One patient with intrapulmonary melanoma exhibited a partial response after HLA-B7 cDNA complexed to cationic liposome was delivered by administration through the right posterior basal pulmonary artery (59). Delivery of antitumor oncogenes by liposomes *in vivo* has also been reported (60). Cationic liposomes themselves may have antitumor effects in certain model systems *in vivo* (61).

Based on the early findings of Nabel *et al.* (62), a number of preclinical tumor vaccine trials or studies designed to augment antitumor immunologic responses using cationic lipids complexed to plasmid DNA have been initiated. In rats, a plasmid incorporating sequences from the adeno-associated virus (AAV) and encoding the IL-2 cDNA was injected into human prostate tumor cells. Direct intramural injection was reported to elicit complete remissions in 50–80% of these animals (63). Liposomes have also been used to efficiently transfer the HSV-tk gene or p53 (64) to pre-established tumors in mice. Although tumor regressions have been reported in these models, the underlying mechanisms of antitumor effects must be reconciled with a relatively low efficiency of gene transfer *in vivo*. A pronounced bystander tumor cell killing effect might be required if the same approaches are expected to promote antitumor responses in patients.

1.8 REGULATORY ISSUES REGARDING LIPID-DNA COMPLEXES

Each formulation of a lipid-DNA complex requires a defined ratio of lipid:DNA and an optimal lipid preparation, usually containing both cationic and neutral lipid components. Preclinical studies can be used to determine the best reagent for a specific purpose. Many phase I type, proof of concept studies, are planned or in progress, and several of these studies include dose escalation. If a clinical study is designed in which a given

formulation will be evaluated at increasing dosages, one set of preclinical safety and efficacy type experiments might be sufficient to support the trial. On the other hand, if a clinical study is designed to modify the composition of a particular formulation (for example, by altering the lipid:DNA ratio), each modification might be viewed as a novel 'drug' from the regulatory standpoint and could require its own complete set of safety and efficacy type preclinical experiments. It may therefore be worthwhile to identify an optimal formulation with a fixed composition early in the preclinical process, and thoroughly evaluate the safety and biologic effect of this particular formulation. Such an approach may simplify the process of initiating human clinical studies.

1.9 SUMMARY

In summary, enormous progress has been made in the development of cationic liposome-based vehicles for gene therapy. Early clinical trials have yielded interesting and, in some cases, encouraging results. Inflammatory changes have generally been less of an issue with these vectors than with adenoviral or other viral constructs studied in the same preclinical or clinical contexts. Low efficiency of gene transfer and short duration of expression are limiting features of the technology. However, because of the enormous flexibility inherent in the strategy, medicinal chemistry approaches to improving the plasmid, lipid or other components of the synthetic complexes support the notion that cationic liposomes may be useful for *in vivo* gene transfer to human patients. Improved understanding of the behavior of these vectors *in vivo* and the mechanisms by which individual cells innately protect themselves from liposome-based transfection are likely to lead to further improvements in the overall technology.

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2 DNA Condensation and Receptor-mediated Gene Transfer

ASSEM ZIADY and THOMAS FERKOL

2.1 INTRODUCTION

The development of strategies to introduce exogenous, functional genes to somatic cells may be important for the future treatment of a variety of human diseases, provided that these methods of delivery are safe, efficient, and selective. Progress in this field has been impressive, and several gene delivery systems are currently being tested in clinical trials. Replication-defective recombinant viruses have been used to mediate gene transfer into a wide range of cell types *in vitro* and *in vivo*. Although recombinant viruses have considerable appeal due to their efficiency, these vectors do have significant practical limitations. For instance, recombinant retroviruses can introduce genes into cells in culture, but these vectors do not transfer genes into non-replicating cells and are generally inefficient in delivering genes *in vivo*. The tropism of specific recombinant viruses can restrict their use, and the packaging capacity of many of these viruses limits the size of foreign sequences that can be inserted into these vectors. Some viral vectors can be cytotoxic or provoke an immune response, thus decreasing effectiveness of gene delivery and transgene expression with subsequent administrations of the recombinant virus. Concerns have also arisen that these disabled viral vectors might become activated following infection with wild-type or helper viruses and produce replication-competent recombinant virus. Because of these considerations, the development of non-viral methods for gene transfer has received considerable attention.

Functional genes can be introduced to eukaryotic cells *in vitro* by a variety of physical, non-infectious methods. Mammalian cells have been transfected by calcium phosphate co-precipitation [1], liposomes [2], DEAE-dextran [3], microinjection [4], gene-particle bombardment [5], and electroporation [6].

Some of these techniques have been extended to transfer DNA into experimental animals *in vivo*. For example, calcium phosphate co-precipitation has been used to deliver DNA into cells grown in culture by exploiting non-specific endocytosis, and this technique has become a standard approach to gene transfer *in vitro*. Investigators have injected calcium phosphate-precipitated plasmids into the peritoneal cavities of rats, resulting in low levels of transgene expression in the liver [7]. Although such transfection methods can transfer DNA into cells, they have been less efficient than viral vectors and the results can be highly variable. In addition, these procedures result in short-lived expression and generally lack specificity, and most of these mechanical techniques have been impractical for delivering genes into animals.

Several non-viral systems, however, have emerged as potential approaches for the introduction of functional genes into animals. Indeed, non-viral methods for gene delivery could have potential for use in human gene therapy if these techniques can be perfected to a dependable and efficient vehicle for gene delivery *in vivo*. In this chapter, we review the development and progress of gene transfer mediated by receptor–ligand endocytosis. Another physical approach, liposome-mediated gene transfer, is reviewed elsewhere in Chapter 1.

2.2 RECEPTOR-MEDIATED GENE TRANSFER

A number of studies have shown that genes have been successfully delivered to cells *in vitro* and *in vivo* by exploiting receptor-mediated endocytosis. The internalization of surface receptors is a ubiquitous cellular response, and many classes of receptors could potentially be used to transfer functional genes into cells. The functions of ligands introduced into cells by receptor-mediated endocytosis are diverse, and the trafficking of the receptors varies. Many of these receptors (e.g. asialoglycoprotein receptor) are designed to deliver their ligands to lysosomes, where they are subsequently degraded. Other receptors recycle their ligands back to the cell surface (e.g. transferrin receptor) or transport their ligand across the cell, where they are released (e.g. polymeric immunoglobulin receptor). Receptor-mediated gene transfer takes advantage of the ability of specific receptors on the surface of cells to bind and internalize large complexes that contain the gene of interest, and provides specificity in the context of a non-infectious and non-toxic vector.

The fundamental design of molecular conjugates that mediate gene transfer has two elements: the ligand and the polycation (Figure 2.1). Construction of the molecular conjugate begins with the selection of an appropriate ligand to target a receptor on a specific cell type. Depending on the receptor, a number of different ligands have been added to the conjugate, including

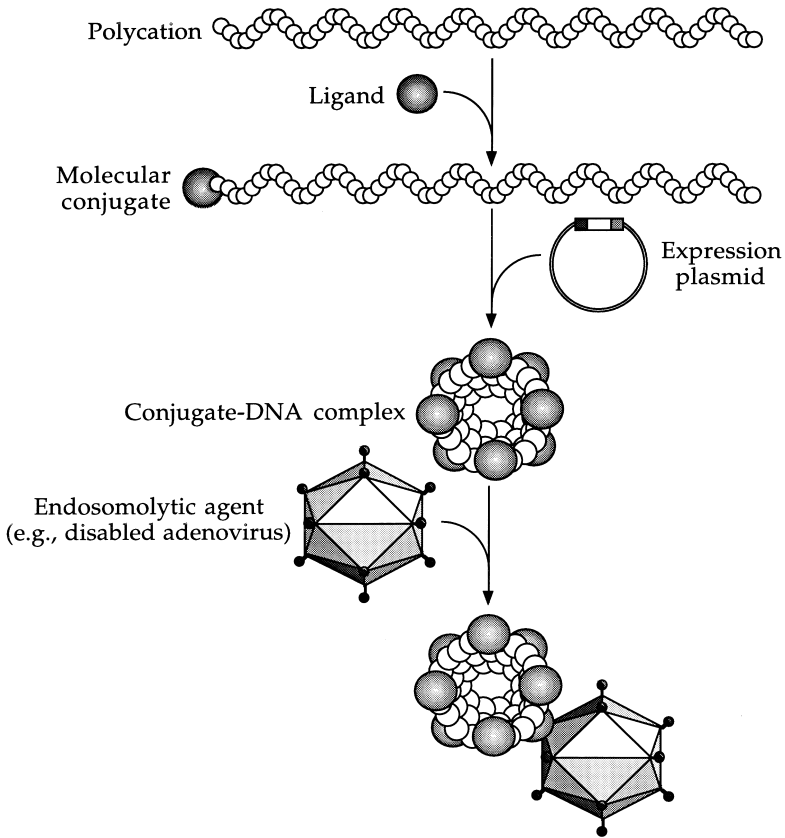


Figure 2.1. Basic structure of transfection complexes. A targeting ligand is covalently linked to a polycation, e.g. poly (L -lysine), which allows the conjugate to non-covalently bind to plasmid DNA and produces a stable conjugate–DNA complex suitable for gene transfer. Endosomolytic agents, like disabled adenoviruses or fusogenic peptides, added to transfection complexes disrupt their endosomal transport and enhance expression of the transgene(s) in the recipient cell.

monosaccharides and disaccharides [8–13], peptides/proteins [14–17], folate [18], glycoproteins [19–21], lectins [22], and antibodies [23–28]. The ligand is covalently linked to a polycation (e.g. polylysine), which interacts electrostatically with the negatively-charged phosphates of the DNA backbone and produces a stable conjugate–DNA complex appropriate for gene transfer. Once the plasmid has entered the cell, it must escape the endosomal compartment in order to transport to the nucleus [29,30]. Consequently, several investigators have incorporated a variety of endosomolytic agents into the molecular conjugate (Figure 2.1).

These molecular conjugates mimic the strategy used by a number of viruses to gain entry into a cell [31]. Nucleic acids are condensed into small particles by polycations, which is similar to the packaging of viral genomes with core proteins. The recognition and internalization of viruses resembles the uptake of molecular conjugates in mammalian cells. For instance, adenoviral proteins bind to receptors (e.g. $\alpha_v\beta_5$ surface integrin) on the host cell's surface, and the virus is subsequently internalized by clathrin-coated pits in a discrete endosome. In contrast to viral vectors, this gene transfer system does not have a packaging constraint and permits the targeting of plasmid DNA of considerable size, allowing for delivery of not only the transgene but its promoter and enhancer elements as well.

2.2.1 TARGETING LIGAND

The novelty of this method of gene transfer is its ability to introduce DNA plasmids into specific cells. Selective targeting of cells may be of great importance for gene therapy, since indiscriminant transgene expression may be disadvantageous. The delivery of exogenous DNA via surface receptors is dependent on several factors, including the presence and number of specific receptors on the surface of the targeted cell, the receptor–ligand affinity and interaction, the stability of the conjugate–DNA complex, and endocytosis of the complex (Figure 2.2). Different cell receptors have been effectively targeted for gene transfer (Table 2.1), and the choice of ligand is critical for the cell specificity of gene transfer. In this section, we review several of the specific receptors that have been used to direct gene delivery.

2.2.1.1 Asialoglycoprotein Receptor

Because of its importance in human metabolism, the liver has long been considered an attractive target for gene therapy, and several receptors expressed in the liver have been used to direct gene transfer to hepatocytes (Table 2.1). In their seminal work, Wu and Wu described a soluble, targetable DNA carrier that delivered expression plasmids to hepatocytes via the asialoglycoprotein receptor pathway [19]. The asialoglycoprotein receptor is an integral membrane glycoprotein that is involved in the clearance of galactose-terminal glycoproteins from the blood by endocytosis through the coated pit/coated vesicle pathway in hepatocytes. Although the majority are transported to lysosomes in hepatocytes, the trafficking of the asialoglycoproteins is leaky [32]. Thus, DNA transferred via this asialoglycoprotein receptor can survive, and because expression of this receptor is limited to hepatocytes, this system potentially has cell selectivity.

To generate a ligand that binds to this receptor, these investigators treated orosomucoid with neuraminidase, which exposed the terminal galactose,

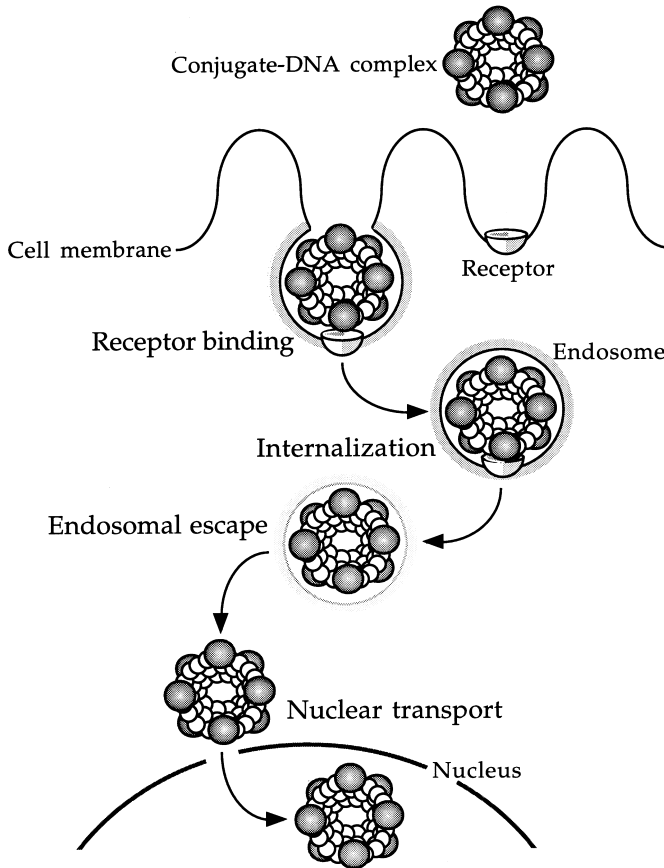


Figure 2.2. Receptor-mediated gene transfer. The foreign gene is condensed into transfection complexes by the molecular conjugate, which binds to a specific receptor on the cell surface. The receptor complex then undergoes endocytosis, and once inside the cell, the DNA escapes from the endosomal compartment and trafficks to the nucleus, where it is expressed. Thus, transgene expression depends on the presence and number of specific receptors on the recipient cell, stability of the conjugate–DNA complex, receptor–ligand interaction, rate of DNA entry, endosomal trafficking and escape, and nuclear transport. In addition, the survival of the transgene and the genetically modified cell, the rate of transcription and translation, and post-translational processing impact on the efficacy of gene transfer.

and covalently linked the desialated glycoprotein to poly-L-lysine. An expression plasmid containing the gene encoding chloramphenicol acetyltransferase was complexed to the molecular conjugate, and the resultant complexes specifically targeted human hepatoma cells (HepG2) that express the asialoglycoprotein receptor [19]. Systemic infusions of conjugate–DNA

Table 2.1 Receptors targeted for gene transfer

Receptor	Ligand	Endocytotic pathway	Target cells	References
Asialoglycoprotein receptor	Asialoorosomucoid Glycosylated polylysine Glycosylated histone Anti-Tn antigen	Lysosomal	Hepatocytes	[9–11, 20, 21, 33–35, 52, 60, 67–69]
Carbohydrate				
C-kit	Anti-CD3 antibody	Lysosomal	Carcinoma cells, lymphocytes Hematopoietic stem cells	[27]
CD				
Cell surface glycoconalx	Steel factor (SLF)	Lysosomal	Lymphocytes	[15]
Epidermal growth factor (EGF) receptor	Lectins	Lysosomal	Ubiquitous	[25]
Folate receptor	Anti-EGF antibody	Lysosomal	Ubiquitous	[22]
Integrins				[28]
Mannose receptor	Folate	Lysosomal	Ubiquitous	[18]
Polymeric immunoglobulin receptor	Peptide	Lysosomal	Ubiquitous	[16]
Serpin–enzyme complex (SEC) receptor	Glycosylated polylysine Anti-pIgR antibody	Lysosomal Transytotic	Macrophages Epithelial cells, hepatocytes	[12, 13] [23, 47, 48]
Surfactant A receptor	Peptide	Lysosomal	Hepatocytes, neurons, macrophages	[17]
	Surfactant A	Lysosomal	Respiratory epithelial cells	[46]
Transferrin receptor	Transferrin	Recycled	Ubiquitous	[14, 36–45, 70–72]

complexes in rats demonstrated the rapid clearance and hepatic uptake of the injected DNA. One day after administration of the conjugate–DNA complexes, chloramphenicol acetyltransferase activity was detected only in the liver, and transgene expression persisted for weeks when the animals underwent subtotal hepatectomy at the time of transfection [20]. Using this approach, Nagase analbuminemic rats received intravenous administration of complexes containing the structural gene for human albumin. The transgene was detected as an episome in the livers of the animals two weeks after treatment, and low levels of human albumin were measured in the blood for four weeks [33].

An asialoorosomucoid-based conjugate has also been used to partially correct other metabolic defects involving the liver. Wilson and co-workers delivered a chimeric gene containing the low density lipoprotein receptor cDNA to the livers of Watanabe rabbits, an animal model for familial hypercholesterolemia [34]. Low density lipoprotein receptor mRNA was isolated one day after administration with the conjugate–DNA complexes, but no transcripts were detected three days post-transfection. The total cholesterol levels in the blood of transfected rabbits were also reduced by thirty per cent two days after treatment, but the effect was transient and blood cholesterol concentrations returned to pretreatment levels by five days after transfection.

Stankovics and co-workers reported that an expression plasmid containing the gene encoding the methylmalonyl CoA mutase, bound to an asialoorosomucoid–polylysine molecular conjugate, increased the enzyme's activity in the liver to a level that could be therapeutic in patients with the inborn error of metabolism, methylmalonic aciduria [35]. Expression was short lived, lasting less than two days after treatment. Injections with the molecular conjugate and conjugate–DNA complexes into mice elicited an antibody response against the ligand, asialoorosomucoid, which may limit its use in chronic diseases [35]. No serologic response to the plasmid DNA was detected in the mice after injection, which is encouraging, since antibodies directed against DNA could preclude such an approach for gene transfer.

Other investigators have used alternative ligands to successfully target the asialoglycoprotein receptor. Molecular conjugates consisting of lactosylated polylysine [9], galactosylated histones [10], and galactosylated albumin–polylysine [21] have been used to transfer reporter genes to hepatoma cells *in vitro*. Recent work by Perales and colleagues has shown that a molecular conjugate consisting of a polylysine chemically modified with α -D-galactopyranosyl phenylisothiocyanate could introduce functional genes to hepatocytes *in vitro* and *in vivo* [11]. An expression plasmid containing the human factor IX cDNA condensed with the galactosylated polylysine was injected into the systemic circulation of rats. The transgene was specifically introduced into the livers of adult animals by receptor-mediated uptake, which

resulted in prolonged expression of the transgene. Human factor IX mRNA and functional protein was detected for weeks after administration of the conjugate–DNA complex [11]. Similar results have been found in Watanabe rabbits injected with complexes containing cDNA encoding the human low density lipoprotein receptor. Although the results have been variable, total cholesterol levels were decreased in the blood of several of the treated rabbits for weeks after injection (M. Molas, J.C. Perales, and R.W. Hanson, unpublished observations).

2.2.1.2 Transferrin Receptor

Another potential target for gene transfer is the transferrin receptor, a dimeric glycoprotein 180 kDa in size. This receptor binds to its natural ligand, transferrin, rapidly internalizes the complex, then recycles the ligand back to the cell surface. The transferrin receptor is ubiquitous, present on the cell membranes of most or all dividing eukaryotic cells, including erythroblasts, hepatocytes, and tissue macrophages; this endocytotic pathway has been exploited to deliver drugs and toxins to tumor cells *in vitro*, as reviewed by Wagner and colleagues [36].

Birnstiel and associates have shown that expression plasmids could be efficiently delivered via the transferrin receptor to avian and human erythroid cells *in vitro* [14,37,38]. Other cell types grown in primary culture have also been successfully transfected. DNA uptake by this receptor was highly efficient, and transgene expression in some of the cell lines could be augmented by co-treatment with lysosomotropic agents. The transfer and expression of reporter genes (*P. photinus pyralis* luciferase) were blocked by the addition of excess free transferrin to the medium, and enhanced by treatment of the host cells with agents (e.g. desferrioxamine) that increase their expression of the transferrin receptor [39]. The addition of inactivated adenovirus particles to the conjugate–DNA complexes also augmented transgene expression [40]. The size of plasmid had little effect of the efficacy of transfer. Cotten and co-workers reported that expression plasmids as large as 48 kilobases could be effectively transferred into cells via the transferrin receptor [41]. More recently, this same group has been able to transfer bacterial artificial chromosomes 160 kilobases in size with an efficiency comparable to that of smaller plasmids (A. Baker and M. Cotten, unpublished observations). The size of complexes formed with the transferrin-based conjugates was critical for gene transfer, and complexes less than 100 nm in diameter were optimal for gene delivery [42].

The transferrin receptor has been used as a target for gene transfer into intact animals, but systemic injection of these transfection complexes, including those containing an inactivated adenovirus, has had limited success, as reviewed by Cotten [43]. It is possible that the inflammatory responses

associated with adenovirus entry interfered with the survival of transfected cells. These same immune responses, however, could potentially be useful in a vaccine application. Zatloukal and colleagues have used the transferrin-based conjugate to deliver the interleukin-2 (IL-2) gene to murine melanoma cells, and high levels of the cytokine were produced in culture [44]. Based on this data, tumor cells transfected *ex vivo* have been re-implanted in animal models and act as a vaccine by inducing an immune response against the tumor. Phase I clinical trials are under way examining the use of this procedure in patients with malignant melanoma.

Local injections of transferrin-based conjugate–DNA complexes into the liver have resulted in high levels of reporter gene expression [43]. Airway epithelia of intact animals have also been transfected using human transferrin–polylysine and transferrin–adenovirus–polylysine molecular conjugates. In these experiments, Gao and colleagues used transferrin or disabled adenovirus as both a ligand and an endosomolytic agent [45]. Intratracheal instillation of DNA bound to these conjugates resulted in transient expression of the reporter gene, which peaked one day after transfection and returned to pretreatment levels by seven days.

2.2.1.3 Polymeric Immunoglobulin Receptor

The lungs play a pivotal role in a number of genetic and non-genetic diseases, and have been proposed as potential organs for gene therapy. Several viral methods of gene transfer have been shown to effectively introduce functional genes into cells of the respiratory tract in animals. Unfortunately, these approaches have had limitations, and alternative techniques for the delivery of genes to the lung are currently being developed. Gene transfer to the respiratory epithelial cells has been mediated by different receptors [45–47], including the polymeric immunoglobulin receptor (Table 2.1).

The polymeric immunoglobulin receptor is specifically adapted for the internalization and nondegradative transport of macromolecules (e.g. dimeric immunoglobulin A and pentameric immunoglobulin M) across epithelia and hepatocytes, and appears to be well suited for receptor-mediated gene transfer. In particular, the distribution of this receptor in humans in airway epithelium and the serous cells of the submucosal glands has led to speculation that this receptor may be an attractive target for the treatment of lung diseases, like cystic fibrosis.

Expression plasmids non-covalently bound to the Fab fragment of antibody directed against the human secretory component, the ectoplasmic domain of the receptor, have been introduced into human tracheal epithelial cells. Ferkol and colleagues have shown that targeting the polymeric immunoglobulin receptor permitted the specific delivery and transient expression of reporter genes to cells that expressed the receptor *in vitro*, even in the

presence of an excess of the receptor's natural ligand [23]. The delivery of expression plasmids, however, was inhibited by the addition of excess human secretory component, which presumably occupied the recognition site on the Fab fragment and prevented its interaction with the receptor. The transfection efficiency of the human tracheal epithelial cells grown in primary culture was variable, though much of this variability appeared to be related to differences in the level of receptor expression.

The polymeric immunoglobulin receptor introduced expression plasmids to tissues that express the receptor *in vivo*. In contrast to the targeting of airway epithelial cells via the transferrin receptor, the conjugate-DNA complexes were administered into the systemic circulation because the polymeric immunoglobulin receptor is predominantly located on the basolateral surface of epithelial cells. Using luciferase and *Escherichia coli* (lacZ) β -galactosidase as reporters, the pattern of the transgene expression also conformed to the spatial distribution of cells that express the receptor [47]. Expression was transient, lasting less than twelve days after injection. A substantial serologic response, however, was directed against the Fab portion of the complexes, which was associated with reduced delivery of the complexes, as evidenced by decreasing transgene expression in the lung and liver after subsequent treatments [48]. Clearly, these immunologic obstacles must be dealt with before such vectors can be used clinically.

2.2.1.4 Mannose Receptor

Tissue macrophages are another potential target for genetic modification, and gene transfer has been directed to these cells via the mannose receptor. The mannose receptor is abundantly expressed by a variety of macrophage subtypes, and internalizes glycoproteins with mannose, glucose, fucose, and *N*-acetylglucosamine residues in exposed, non-reducing positions. Like the asialoglycoprotein receptor, ligands internalized by the mannose receptor are trafficked to lysosomes. Synthetic glycoprotein conjugates have been constructed in which polylysine was glycosylated with various monosaccharides, and used for receptor-mediated gene transfer to murine and human macrophages in primary culture [12,13]. The transfection efficiency varied considerably, but transgene expression co-localized with monocyte/macrophage markers [12,13]. The uptake of the reporter gene was mediated by the specific interaction of the complex with the mannose receptor, since the addition of excess mannosylated bovine serum albumin to the culture media before transfection inhibited expression of the reporter gene [12].

Expression plasmids, complexed to the mannose-terminal glycoprotein conjugate, have also been introduced into tissue macrophages in rodents through the mannose receptor. These complexes were injected into the systemic circulation of adult mice [12]. Reporter genes were successfully de-

livered to reticuloendothelial organs in mice, though transfection efficiency was low and transgene expression was short lived, peaking four days after transfection.

2.2.2 DNA CONDENSATION AND COMPLEX FORMATION

In initial reports, gene transfer mediated through the asialoglycoprotein receptor showed little dependence on the size of the condensed DNA particles. Indeed, the polycation was simply viewed as a method to adhere the ligand to the plasmid DNA. The method of condensation used in these experiments involved techniques in which DNA is annealed to excess polycation, then subjected to dialysis against a sodium chloride gradient. The DNA was gradually condensed into multimolecular positively-charged particles with an apparent size distribution averaging about 150–200 nm in diameter [19].

The size of the conjugate–DNA complexes may be important for gene transfer since many endocytotic receptors discriminate against ligands of a determined size range. Wagner and co-workers have shown that the degree of condensation of the complexes correlated with the level of transgene expression. Specifically, conjugate–DNA complexes condensed into toroid structures measuring 80–100 nm in diameter were most effective for gene transfer into cells grown in culture [42]. Perales and colleagues have developed an alternative method of condensing expression plasmids into compact particles that are suitable for gene transfer, and have determined the conditions that are important for the stabilization of these complexes formed at high concentration of DNA [11]. This system involves the gradual addition of small aliquots of the ligand–polycation conjugate to plasmid DNA. By adjusting the concentration of sodium chloride in the solution, ‘unimolecular’ complexes measuring as little as 10–20 nm in diameter by electron microscopy were produced. The complexes had a net neutral surface charge, which may avoid complement activation and provide for a more efficient vector for gene transfer into animals [49].

The degree of DNA condensation of the conjugate–DNA complex is dependent on several variables, including the concentration of sodium chloride, length of the polylysine, and the size, sequence, and physical state of the DNA [11]. The length of the polylysine affects sodium chloride concentration necessary to form and maintain these complexes. The secondary structure of the polycation could also influence the structure of the complexes. Synthetic peptides arranged in different conformations (i.e. random coil, α -helix, and β -sheet structures) and bound to DNA produced multimolecular aggregates that may be unsuitable for receptor-mediated uptake [50]. Finally, construction of the molecular conjugate affects its binding to DNA; the interaction of polycation with DNA can be destabilized by increased substitution with

ligand. Extensive substitution of the polycation lessens its affinity to DNA, probably by steric hinderance, with the result being a less tightly packed complex, which may be too large for internalization. Genes were more effectively transferred via the serpin–enzyme complex *in vitro* with the less substituted polycation. Poly-L-lysine alone, however, produced the smallest complexes but failed to transfect cells. In addition, the pattern and duration of transgene expression varied depending on chain length of polycations, even though the different molecular conjugate gave the same degree of DNA condensation. (A. Ziady, T. Ferkol, and P.B. Davis, unpublished observations). Wagner and colleagues discovered that molecular conjugates with fewer ligand moieties are more effective at delivering reporter genes by receptor-mediated endocytosis [42]. Conjugates containing approximately 1 transferrin per 100 lysine residues resulted in maximal transgene expression in human erythroid cells. Moreover, the partial replacement of the transferrin-based conjugate with free polylysine produced smaller toroidal structures and higher levels of luciferase activity [42].

The condensation of plasmid DNA by a polycation may have additional advantages, since tightly compact DNA may be important for its stability in the blood and in the target cell's cytoplasm. High molecular weight DNA is particularly susceptible to shearing by hydrodynamic forces, and it has a remarkably short half-life in the blood, presumably due to its digestion by nucleases [51]. The polycation can influence the survival of the exogenous DNA in cytoplasmic endosomal vesicles, because plasmid DNA condensed with polylysine appears to be more resistant to endonuclease digestion [52]. Several different polycations have been used to transfer genes. The most widely used DNA condensing agent is poly-L-lysine, but poly-L-ornithine and poly-L-arginine have been used to transfect cells *in vitro* [53].

Interestingly, plasmids non-covalently linked to poly-L-arginine were poorly expressed, which may be related to the observation that poly-L-arginine binds to DNA with greater affinity than poly-L-lysine and may prevent transcription factors from interacting with the transgene (A. Ziady, T. Ferkol, and P.B. Davis, unpublished observations). Poly-L-amino acids may have additional advantages as vehicles for gene transfer. Poly-L-amino acids are non-antigenic and are promptly destroyed and metabolized [54,55]. In contrast, peptides consisting of D-isomers of amino acids are relatively resistant to degradation [56]. Finally, it has been postulated that poly-L-lysine could serve as a nuclear localization signal and may be more efficient in targeting the conjugate–DNA complex to the nucleus once it has been internalized [57].

A variety of synthetic cationic polymers have been shown to mediate gene delivery into cells. Cascade polymers, otherwise known as starburst dendramers, have transferred reporter genes to different cell types *in vitro* [58]. Producing complexes with a net positive charge, Szoka and co-workers

achieved optimal transfection efficiency using dendramers 6.8 nm in diameter, and conjugation of the cascade polymer to a membrane-destabilizing protein further enhanced gene delivery. Polyethylenimine, a highly branched organic polymer synthesized by polymerizing aziridine, employed as both a DNA-binding protein and a lysosomal disruption agent, has been used by Boussif and colleagues to successfully introduce plasmids containing the luciferase reporter gene into cells of the central nervous system [59]. Indeed, polyethylenimine acts as a 'proton sponge' in the endosome.

Other DNA-binding molecules have been used to link expression plasmids to ligands and deliver genes to mammalian cells. Nucleoproteins, like histones [10,42] and protamine [26,37], have been employed as the polycation in several molecular conjugates. The intercalating agent bisacridine, chemically modified to contain terminal galactose residues, has been used to transfer DNA into cells expressing the asialoglycoprotein receptor [60]. Using a novel variation, Fominaya and Wels exploited the high affinity of the yeast transcriptional activator GAL4 for specific DNA recognition sequences to bind expression plasmids to a molecular conjugate and transfect cells *in vitro* [61].

The size and conformation of DNA may be important variables for gene transfer, yet plasmid DNA does not necessarily need to be condensed into compact particles for uptake in some tissues to occur. Wolff and colleagues have demonstrated that direct injections of pure DNA into muscle result in prolonged expression of reporter genes in myofibers [62,63]. The plasmids remain extrachromosomal in the host cell's nucleus, and persist for months as non-replicating episomes. Although other cell types have been successfully transfected using this approach [64–66], this phenomenon appears to be relatively specific to muscle. Thus, the application of direct DNA injections may be limited.

2.2.3 ENDOSOMAL TRAFFICKING AND ESCAPE

Non-viral vectors that introduce transgenes into the cell by receptor-mediated endocytosis are simply a means to penetrate the barrier posed by the cell membrane and permit the introduction of exogenous genes to the targeted cell's cytoplasm. Expression of the transferred genes requires their escape from the endosomal vesicles and trafficking to the nucleus [29]. In the case of the asialoglycoprotein receptor, the trafficking of the ligand appears to be degenerate. Although the majority of galactose-terminal glycoproteins are transported to lysosomes in primary rat hepatocytes, their degradation is incomplete and some of the internalized asialoglycoproteins avoid destruction. The conjugate–DNA complexes introduced via this receptor may similarly escape destruction. Although conjugate–DNA complexes imitate the entry processes of viruses, they are inefficiently released from the endosomal

compartment. In fact, transgene expression appears to be limited by trapping or degradation of the complex in endosomes (Figure 2.2). The efficiency of gene transfer through receptor-mediated endocytosis could be improved by interrupting endocytic trafficking of the conjugate–DNA complexes. It is possible that the presence of the polycation in the molecular conjugate permits the escape of the conjugate–DNA complexes from the endosome. Cotten and associates have shown that chloroquine, a lysosomotropic agent that interferes with the acidification of lysosomes and inhibits hydrolytic enzymes, greatly improves the expression of transgenes in human erythroid cells (K562 cell line) transfected with the transferrin–polylysine conjugate [39]. Chloroquine has also been shown to augment the expression of transgenes delivered to other cell types, like hepatoma cells and human macrophages, by receptor-mediated endocytosis [9,13]. Another pharmacological agent, colchicine, has been shown to improve the survival of transgenes *in vivo* by interfering with the trafficking of endosomes by disrupting the microtubules [67].

Several investigators have employed adenoviruses to improve the survival of macromolecules by receptor-mediated endocytosis. The regulation of endosomal pH is critical for the appropriate trafficking of ligands along endocytotic pathways, and the pH of the endosomal vesicle becomes more acidic as it routes to the lysosome. Acidification of the late endosomes produces conformational changes in the adenoviral capsid proteins and causes an interaction between the protein and vesicle membrane, thus disrupting the endosome. This observation led to the hypothesis that endosomal disruption caused by the adenovirus could improve the survival and expression of transgenes. Curiel and associates have exploited this property of the virus and have coupled replication-defective adenovirus to transferrin-based molecular conjugates [40]. These adenovirus-linked molecular conjugates greatly enhanced the expression of different transgenes in a variety of cell types *in vitro*. Moreover, reporter genes have been introduced into airway epithelial cells *in vivo* through the intratracheal route using adenovirus–polylysine and transferrin–adenovirus–polylysine vectors [45]. Cristiano and colleagues modified the asialoorosomucoid-based molecular conjugate by the addition of an inactivated adenovirus to the vector, and demonstrated that coupling the adenovirus to the conjugate–DNA complexes augmented the delivery of reporter genes into hepatocytes in culture [68,69]. Alternative viruses (e.g. chicken embryo lethal orphan virus) have also been used to increase transgene expression [70].

Surface proteins from other viruses have had a similar effect on the survival and expression of transferred genes by permitting the fusion of endosomal and viral membranes. Wagner and associates have shown that peptide sequences derived from the influenza hemagglutinin HA-2 bound to the transferrin–polylysine molecular conjugate markedly increased the level

of transgene expression in cells grown in culture [71,72]. Other investigators have constructed synthetic peptides that imitate the endosomolytic functions of viral proteins. Other peptides, like the fusion protein of the respiratory syncytial virus or synthetic endosomal release peptides, may have a similar effect on the release and survival of exogenous DNA [73]. However, the addition of such proteins may have a potential drawback, since linkage of the peptide to the conjugate may add to the immunogenicity of the complexes.

2.2.4 NUCLEAR TARGETING AND TRANSLOCATION

The ultimate goal of gene transfer is the delivery of exogenous DNA to the nucleus, where transgenes can undergo transcription (Figure 2.2). The process of nuclear trafficking of DNA complexes once they escape the endosome, however, is perhaps the least understood process in receptor-mediated gene transfer. Nuclear entry of the conjugate-DNA complexes may be a passive, random process and simply relate to the total number of copies of the transgene present in the cytoplasm. It is possible that the transport of transgenes to the nucleus requires cell division, and that the disintegration of the nuclear envelope during mitosis permits the entry of extrachromosomal DNA. The efficiency of gene transfer via the asialoglycoprotein receptor has been shown to be increased when hepatic regeneration was induced by subtotal hepatectomy [20,21]. Nevertheless, the importance of cell division for the nuclear targeting of the transgene has yet to be determined. The fact that reporter genes can be effectively delivered to non-dividing cells suggests that this is unlikely to be the mechanism. Transgene expression in the liver was also remarkably prolonged in the animals that underwent subtotal hepatectomy, though it is uncertain if the exogenous genes were integrated into the host cell's genome [20,21,33]. Indeed, it is most likely that the majority of DNA transferred by these molecular conjugates in cells and tissues exists as episomes in the nucleus, and will not persist in the nuclei of transfected cells. The prolonged survival of the reporter gene may be related to the transgene escaping the asialoglycoprotein receptor pathway, thus avoiding degradation in lysosomes and allowing the DNA to persist in non-hydrolytic cytoplasmic vesicles.

Another compelling hypothesis is that the polylysine component of the carrier may assist in nuclear trafficking of the DNA. Several viral proteins responsible for translocation of the viral genome to the nucleus are highly basic and have sequences rich in lysine. The amino acid sequence Phe-Lys-Lys-Lys-Arg-Lys-Val has been shown to transport the simian virus-40 (SV-40) large T antigen to the nuclei of mammalian cells [74], and the sequence Lys-Lys-Lys-Tyr-Lys-Leu-Lys serves as a specific nuclear localization signal for the matrix protein for the human immunodeficiency virus type-1 [75].

Nucleoproteins, which are also highly basic, have been employed as the polycation or nuclear localization signal in several molecular conjugates. Specifically, different types of histones and protamine have been used to transfer functional genes. The addition of histone H4 to transferrin-based conjugates was shown to enhance transgene expression in cells *in vitro* [42]. Histone H1, chemically modified to contain terminal galactose residues, was also used to introduce reporter genes into hepatoma cells via the asialoglycoprotein receptor [9]. The mechanisms by which these proteins actually enhance gene transfer still need to be determined.

In this system, the plasmid DNA does not appear to integrate into the host cell genome, and transgenes remain episomal. Consequently, the gene will most likely not persist in the nuclei of transfected cells and be transiently expressed. The duration of transgene expression also appears to vary depending on the tissue or cell type transduced, and may be related to the relative survival of the recipient cells. Obviously, genes introduced into a host are lost when the targeted cells die. The transgene product itself can affect the survival of the genetically modified cell. The expression of certain reporter genes could induce a cellular immune response, which eliminates the transfected cells and limits transgene expression. Exotoxin contamination of plasmid preparations has been shown by Cotten and co-workers to injure primary cells *in vitro* and affects the expression of reporter genes [76]. Thus, the use of pure, pharmaceutical grade DNA is essential.

The loss of the transferred DNA in the host cell is a serious limitation to these systems, and several investigators are designing DNA vectors that should extend the survival of the transgene. Self-replicating episomal vectors that contain viral sequences that act as an origin of replication could allow the transgene to persist in dividing cells [77]. In addition, artificial chromosomes are being developed that should permit the delivery and continued expression of not only the transgene (cDNA or intact gene) but its promoter and enhancer elements as well [78].

2.3 CONCLUSIONS

In summary, a variety of molecular conjugates have been used to transfer exogenous, functional genes into mammalian cells by exploiting receptor-mediated endocytosis. Receptor-mediated gene transfer affords a flexible, non-infectious method for delivering condensed DNA to specific target cells *in vitro*, and the incorporation of endosomolytic agents into the molecular conjugate greatly enhances transgene expression. Despite their potential advantages (Table 2.2), these gene delivery systems have produced variable results *in vivo*. Reporter genes introduced into the liver have resulted in prolonged expression, which appears to be related to the size and conforma-

Table 2.2 Receptor-mediated gene transfer*Advantages*

Non-infectious

Efficiently transfers plasmid DNA to cells *in vitro*

Targeted delivery of foreign genes to cells bearing the appropriate receptor

No size constraints on the foreign DNA transferred

Multiple genes can be delivered to cells

Cell replication not required for transfection or transgene expression

Addition of endosomolytic agents improves survival of transgene

Transgene remains extrachromosomal

*Disadvantages*Less efficient delivering plasmid DNA to tissues *in vivo*

Short-lived transgene expression

Variable, low-level expression of transgene

Ligand or endosomolytic moieties may be immunogenic

tion of the plasmid DNA. Otherwise, genes delivered by molecular conjugates have been transiently expressed in tissues at low levels *in vivo*, despite modifications in the complexes designed to promote the survival of the transgene in the recipient cell.

Several investigators are addressing these limitations by incorporating features of molecular complexes into other gene transfer vehicles, like liposomes. Condensation of plasmid DNA with basic proteins, like polylysine, has been shown to augment gene transfer by cationic liposomes due to improved encapsulation of the DNA and possibly the endocytosis of the complexes [79]. Liposomes tend to fuse indiscriminately with cells, and the addition of a ligand to the lipid–DNA complex may permit the selective delivery of genes to the targeted cells [80].

Chimeric vectors are also being designed to combine elements of both viral and non-viral systems. Several investigators have already included viral proteins into molecular conjugates to enhance the delivery and expression of transgenes. The converse should also be true, and recombinant viruses could be modified to include ligands, which should permit their targeting to specific cells that express the receptor. An ecotropic Moloney murine leukemia virus that was chemically modified with lactose targeted the asialoglycoprotein receptor on the surface of HepG2 hepatoma cells [81]. Alternatively, Kasahara and colleagues have genetically engineered the envelope of an ecotropic retrovirus to contain erythropoietin, and this hybrid vector was used to deliver transgenes to murine and human cells that express the erythropoietin receptor [82]. Avian retroviruses have also been modified to express an integrin on their surface and used to infect eukaryotic cells [83]. These chimeras could potentially permit the tissue-specific targeting of recombinant viruses by the interaction of ligands with cell surface receptors, and may represent the future of receptor-mediated gene transfer.

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3 Retroviral Vectors

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3.1 INTRODUCTION

Viruses are obligate parasites and cannot replicate without the help of host cells. Thus they are, by nature, adapted to efficiently transmit genetic information to cells. Among the viruses many different strategies have evolved that allow them to enter cells, and the form of their genetic information to some extent determines the later mechanisms used for replication and production of new virus particles or virions.

Retroviruses are particularly of interest in this respect, because although they carry an RNA genome, there is a requirement that this genome be transcribed into a double-stranded DNA form in the infected cell, which can then be efficiently integrated into the host cell DNA. Once integrated, the virus DNA, known as a provirus, is transcribed like any other cellular gene and the virus-specific RNA is used to produce virus proteins and new genomic RNA, both of which are assembled into new virions. The integrated provirus is a stable part of the cell genome for the life of the cell and is passed on to all daughter cells that arise from the original infected cell. This latter property of retroviruses makes them ideal candidates for gene delivery vehicles, also known as vector systems, that give long-term gene expression.

In addition to the 'vector-friendly' properties of the retroviral life cycle, the knowledge gained from over 40 years of intensive research means that, in comparison to other viruses, the biology of these viruses and their interaction with the host cell is very well understood. It was realised very early on that these viruses can transmit cellular genes (Stehelin *et al.*, 1976) and consequently they were used as the first viral vector system. Although these early vector systems were very unsophisticated, relying on wild-type virus to transmit the recombinant genome carrying a marker gene (Shimotohno and Temin, 1982; Tabin *et al.*, 1982), improved systems have been created since and their evolution to ultimately create the perfect vector is still proceeding. Before discussing some of these systems, it is necessary to outline briefly the

salient features of the replicative cycle of retroviruses and how vectors are derived from these viruses.

3.2 THE RETROVIRAL REPLICATION CYCLE

The retroviral life cycle is summarised in Figure 3.1.

3.2.1 EARLY EVENTS

Retroviruses are enveloped viruses that carry two identical copies of a single-stranded RNA genome in the virus particle (for a review of retroviruses see Varmus and Brown, 1989). The outermost viral envelope protein (surface or SU protein) binds specifically to defined receptors that extend out of the target cell plasma membrane, triggering virus uptake by the cell (Figure 3.1). After release of the inner viral capsid from the viral envelope (uncoating), the viral genomic RNA within the capsid is converted into a double-stranded DNA form by the capsid-associated reverse transcriptase (RT) enzyme. This process initiates using a tRNA primer that is found in the capsid, specifically bound to the retroviral genomic RNA. During reverse transcription, unique sequences at either end of the viral genomic RNA are duplicated and placed at both ends of the newly synthesised DNA, generating a relatively long repeated sequence at each end of the DNA molecule, termed a long terminal repeat (LTR). The double-stranded DNA copy, flanked by the two LTRs, is then translocated to the nucleus (Figure 3.1).

3.2.2 INTEGRATION OF VIRAL DNA INTO HOST CELL GENOMIC DNA

After arriving in the nucleus, the double-stranded DNA form is integrated into the host cell chromosomal DNA by the virally associated integrase (IN) enzyme (Figure 3.1). The integrated DNA form is termed a provirus. Integration of the provirus is essentially random with respect to the host cell chromosomal DNA, although there may be some preference for actively transcribed regions. There are two major features of retroviral integration that are also relevant for gene therapy applications. First, the provirus is always found as a co-linear DNA with the structure LTR–retroviral genes–LTR. This is in sharp contrast to other integrated DNAs resulting from infection with other viruses or from naked DNA transfer, in which the transferred DNA is present in a permuted form. Second, the provirus is stably inherited by all the offspring or daughter cells of the originally infected cell, as if it were a normal cellular gene, usually without apparent deleterious effects. This contrasts with other viruses such as adenoviruses or

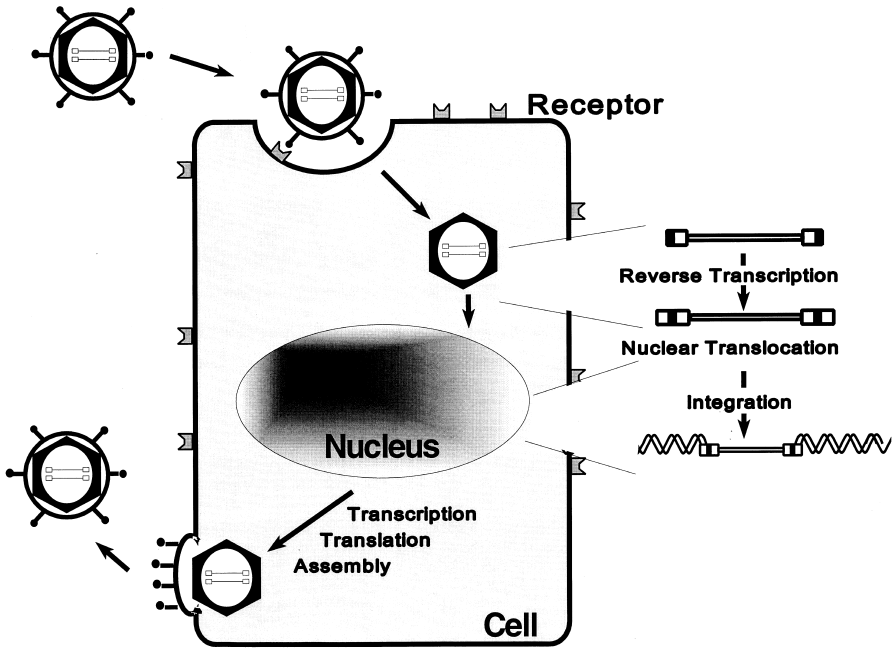


Figure 3.1. The replication cycle of a retrovirus. Retroviruses are enveloped viruses that carry two identical copies of genomic RNA. In order to infect a cell, the virus SU envelope protein (circles on stalks) interact with cell encoded receptors that are displayed on the plasma membrane. This interaction leads to the uptake and internalisation of the virus particle and the loss of the virus envelope, which occurs by membrane fusion. The viral genomic RNA is used as a template for the production of a double-stranded DNA molecule by the reverse transcriptase enzymatic activity, present in the core (shown in the upper inset). During this process, sequences at either end of the viral genome are duplicated and translocated (see also Figure 3.6). The complex then translocates to the nucleus, where the double-stranded DNA molecule is inserted into the host cell genomic DNA by the viral integrase enzyme (shown in the lower inset). The integrated DNA form of the virus genome, known as the provirus, is then transcribed like any cellular gene by the host cell transcription machinery from the viral promoter. Newly transcribed viral RNAs are used for the translation of new virus proteins and also as the genomic RNA for virus particles. The virus proteins and RNA assemble into new cores or capsids, which interact with regions of the host cell membrane that contain the newly synthesised virus envelope proteins, and newly produced virus particles bud out of the infected cell, where they undergo the final stages of maturation to form fully infectious virus particles.

pox viruses, which do not integrate their genetic information and which kill the successfully infected cell.

Retroviral infections in animals are often associated with tumour induction, notably leukaemias. This is a result of multiple integration events eventually leading to integration into, or in the vicinity of, cellular genes involved in growth control (proto-oncogene or tumour suppressor gene), thereby resulting in their dysfunction, a process known as insertional mutagenesis. This requires actively replicating (replication-competent) retrovirus capable of multiple successive integrations. Retroviral vectors are replication defective (see below) and only integrate once. The chance of a single retroviral integration occurring in such a gene locus is thus extremely low. Even if such a cellular gene were affected by retroviral integration, other genetic lesions would be required before a cell could be transformed to a malignant state. There is no evidence of retroviral integration causing tumours in humans. Nevertheless, it is imperative that no replication-competent virus is present in clinical stocks of retroviral vectors, and considerable effort has been devoted to this issue.

3.2.3 TRANSCRIPTION, TRANSLATION AND ASSEMBLY

Transcription of the integrated provirus is directed by the viral promoter and enhancer elements, located in the 5' (left-hand) LTR and terminated in the 3' (right-hand) LTR. Retroviruses carry three major genes which encode the viral core proteins (*gag* gene) forming the inner structure of the virus, the enzymes reverse transcriptase and integrase (*pol* gene) and the viral envelope proteins (*env* gene). The primary genomic length transcript carries all of the viral genetic information and is used both as the genome for new virions and also for the production of the Gag and Pol proteins. A second spliced transcript is used for the synthesis of the viral Env proteins. Some retroviruses, notably lentiviruses such as human immunodeficiency virus (HIV), produce other spliced transcripts, giving rise to accessory proteins that regulate virus protein production.

The Gag and Pol proteins are translated as polypeptide precursors from the genomic length transcripts and sequentially proteolytically cleaved into the mature proteins. Cleavage is an ongoing process that occurs as the virus assembles, buds from the host cell and matures outside of the host cell. Domains of the precursor proteins specifically interact with viral RNA (Zhang and Barklis, 1995), ensuring that viral rather than cellular RNA is packaged within the progeny virus. This interaction requires one or more specific RNA sequences, known as packaging signals, on the genomic RNA, some of which are obligatory (ψ) and located at the beginning of the Gag region, just 3' of the splice donor (SD) for subgenomic RNA production (see below), ensuring that only full-length viral RNAs can be packaged.

The Env proteins (SU and TM) are translated from a spliced subgenomic viral RNA. These proteins are synthesised as a precursor which carries a rapidly removed signal peptide at the amino terminus, and thus enters the secretory membrane system of the host cells. During its passage through the secretory system, the precursor becomes modified by a number of glycosylation steps and is finally cleaved into the mature SU and TM proteins by a cellular protease. However, both proteins remain associated with each other by disulphide bonds and finally arrive at the host cell plasma membrane.

The final steps of assembly and release of progeny virions occur at the cell membrane, where the Gag proteins associated with the retroviral genomic RNA and viral RT and IN enzymes form new capsid structures (Figure 3.1). The capsid buds through the cell membrane in areas where the concentration of inserted viral Env proteins is relatively high. The newly released virions undergo further maturation steps associated with protein cleavage, conformational changes and reorganisation of viral proteins within the core to give the final infectious progeny virions. It should be stressed that for most retroviruses the whole process of retroviral infection and virus production does not harm the producing cell in any obvious way.

3.3 RETROVIRAL VECTOR DEVELOPMENT

The first retroviral vector systems were derived from murine leukaemia virus (MLV) and these vectors are the ones that have and are being used in clinical trials. There were (and are still) a number of reasons for choosing MLV as the basis for such gene delivery systems, including (i) the biology of this retrovirus is particularly well understood, (ii) the MLV genome was among the earliest retroviral genomes molecularly cloned and (iii) these viruses are able to infect cells efficiently. MLVs exist that are able to infect only rodent cells and these are termed ecotropic. The ecotropic viral SU protein interacts with an amino acid transporter protein that is found in the plasma membrane of target cells (Kim *et al.*, 1991; Wang *et al.*, 1991). The murine form of this transporter functions as a virus receptor whereas the human form does not due to critical amino acid differences. Thus, ecotropic MLV is not able to infect human cells unless it is engineered to do so. A different MLV variant is able to infect many cell types, including human and rodent cells. The SU protein of this amphotropic virus interacts with a cellular phosphate transporter protein (Miller and Miller, 1994; van Zeijl *et al.*, 1994). Amphotropic MLVs are used in many of the ongoing gene therapy trials. The envelope of gibbon ape leukaemia virus (GaLV) has also been used since GaLV envelope carrying retroviral vectors gives better haematopoietic cell gene transfer efficiencies (Bunnell *et al.*, 1995).

3.3.1 PRINCIPLES

Retroviral vector systems consist of two components: (i) a vector construct that carries the gene to be delivered and provides the genome for the recombinant virus, and (ii) a cell line that provides the viral proteins required to produce the recombinant virus, known as packaging cells (Figure 3.2). Two-component systems originally arose because insertion of additional genetic information into the MLV genome is detrimental to virus production, necessitating the deletion of structural gene coding sequences and the provision of these proteins *in trans* by packaging cells. Even though it may be possible to create one-component replication-competent retrovirus vectors (for example based on HIV or Rous sarcoma virus), it seems unlikely that this approach will be pursued, given the concerns about retroviral-mediated insertion mutagenesis (see Section 3.2.2).

To produce recombinant retroviral vector virions, the vector construct carrying the gene(s) to be delivered is introduced by physical gene transfer methods (such as transfection, electroporation etc.) into a retroviral packaging cell line. These packaging cells produce the viral structural (Gag and Env) proteins and enzymes (*pol*-encoded RT, IN), but are not able to package the viral RNA encoding these proteins since the ψ region required for encapsidation has been deleted. Instead the proteins recognise and associate with genomic length RNA from the introduced vector construct, which carries an intact ψ region, to form recombinant virus particles. The recombinant virus particles carrying the retroviral vector genome bud out of the packaging cell line into the cell culture medium. The virus-containing medium is either directly filtered to remove cells and cellular debris and then used to infect the target cell, or virus is purified and concentrated before infecting target cells. After the virus has bound to the receptor on the cell surface, the viral capsid is delivered into the cell and the viral RNA is reverse transcribed into a DNA form which integrates into the host cell DNA. The integrated viral DNA (provirus) functions essentially as any other cellular gene and directs the synthesis of the products of the delivered gene(s). However, unlike in the case of normal wild-type replication-competent virus (described in Section 3.2), no further infectious virus can be produced by the infected cell since the genetic information encoding the viral proteins is not present in this cell (Figure 3.2).

The major problem with two-component retroviral vector systems arises as a result of the naturally occurring phenomenon of homologous recombination. If the vector provirus and the provirus providing the structural proteins in the packaging cells recombine, there is a possibility that replication-competent retrovirus will arise (Figure 3.3; Miller and Buttimore, 1986; Muenchau *et al.*, 1990). Such virus is essentially a wild-type retrovirus and no longer carries the delivered gene(s). Replication-competent virus rapidly infects many cells and may eventually cause insertional mutagenesis. Conse-

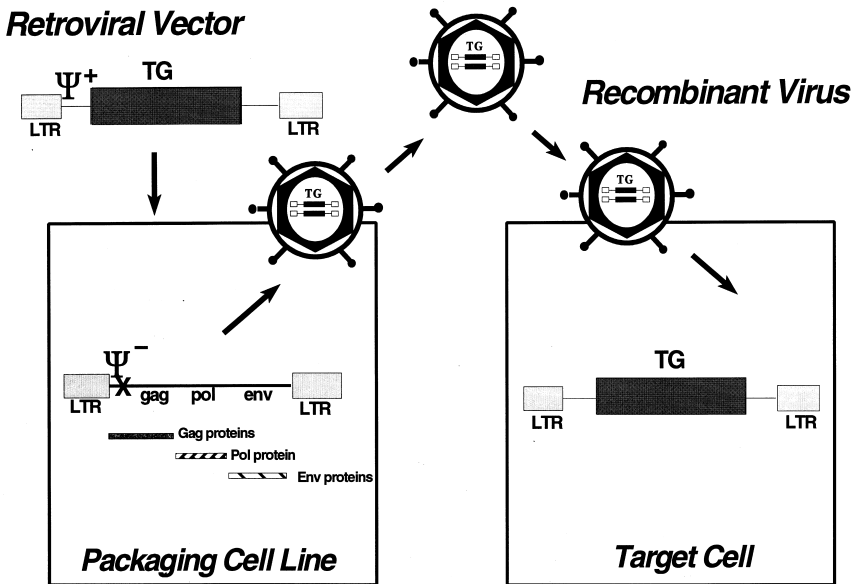


Figure 3.2. Principle of retroviral vector production. The vector construct consists of a provirus that carries a gene(s), for example a therapeutic gene (TG), in place of viral genetic information. The gene is placed under the transcriptional control of, for example, the viral promoter located in the long terminal repeat (LTR). The vector is introduced into a cell line (packaging cell line) which carries a modified retroviral provirus. This provirus produces the virus proteins that the vector cannot produce, but because it lacks the appropriate ψ packaging signal (Ψ^-), cannot insert its own genome into the virus particles produced. The vector construct carries the ψ packaging signal, and thus RNA transcribed from this construct is preferentially inserted into the newly formed virus particles that are constantly being released from the packaging cells. The virus particles carrying the vector genome are then used to infect target cells, leading to reverse transcription and eventual integration of the vector genome carrying the therapeutic gene (TG). This gene is then expressed, giving therapeutic protein production in the target cell. No further virus can be produced since no viral structural proteins are present in these cells.

quently, considerable effort has been devoted to the design of superior packaging systems that drastically reduce the possibility of recombination occurring, as well as to produce improved, safer vectors that cannot replicate even if recombination occurs.

3.3.2 IMPROVEMENTS

Improvements to packaging cells have involved removing as much of the retroviral information as possible to reduce the possibility of homologous recombination occurring (Figure 3.4). The retroviral promoter and termina-

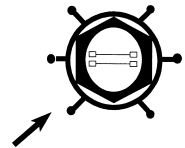
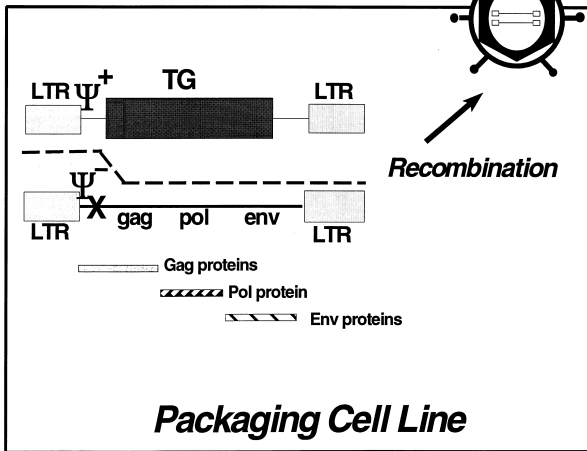
Retroviral Vector**Wild Type Virus**

Figure 3.3. Generation of replication competent (wild-type) virus by recombination. Initial retroviral vector systems often rapidly became contaminated with replication-competent virus due to at least one homologous recombination event (dotted line) occurring between the vector construct and the packaging construct in the packaging cells. The probability of this happening depends on the degree of homology shared between the two constructs.

tion sequences can be replaced by heterologous promoters and termination sequences. This has the additional advantage of allowing the use of promoters that are more strongly active than the retroviral promoter, thereby giving rise to higher levels of viral protein production. The coding information for the viral proteins cannot be removed by necessity, but these proteins can be made from separate constructs so that additional recombination events are required to recreate a complete replication-competent retrovirus. This has been achieved by expressing the Gag and Pol proteins from one construct and the Env proteins from a second construct (Markowitz *et al.*, 1988a,b).

In addition to the improvements to packaging cells, safer retroviral vector constructs also have been produced that carry an artificially inserted stop codon in the Gag reading frame. This ensures that even if replication-competent virus is generated, it will not be able to express its Gag and Pol proteins and thus virus assembly and release will be inhibited (Bender *et al.*, 1987; Morgenstern and Land, 1990; Scarpa *et al.*, 1991). Another strategy for

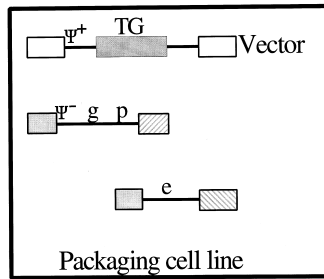


Figure 3.4. Improved retroviral vector system. In order to reduce the possibility of homologous recombination leading to the production of replication-competent retrovirus, systems have been constructed in which only those viral sequences that are absolutely required are maintained in the vector and packaging constructs. This has been facilitated by the use of heterologous promoters (open and shaded boxes) and termination signals (striped boxes). In addition, such systems consist of three components, for example two independent constructs, one of which expresses the Gag (g) and Pol (p) gene products and a second that is used for expression of the Env (e) proteins. At least three recombination events between the vector and the two packaging constructs are required for the production of replication-competent virus.

creating a fail-safe mechanism to prevent the infectivity of any potential replication-competent virus is to ensure that the packaging sequence becomes deleted. This can be achieved by flanking the ψ sequences with direct repeats, facilitating their removal. The majority of vector proviruses in cells infected with such vectors were shown to have deleted the packaging sequences, presumably due to template switching during reverse transcription (Julias *et al.*, 1995). The Cre/loxP recombinase system has also been incorporated into retroviral vectors and used to specifically excise viral vector sequences in the infected target cell (Cholika *et al.*, 1996; Russ *et al.*, 1996). Vectors have also been constructed that carry a modified, artificial primer binding site (PBS) (Lund *et al.*, 1997). Normally a cellular tRNA is bound to this region of the genomic RNA and it is used as the primer for initiation of reverse transcription. In combination with a packaging cell line which also synthesises the artificial tRNA, recombinant virus can be produced and used to infect cells. However, any replication-competent virus that may be produced from this system as a result of homologous recombination will not be able to replicate after the initial infection of a cell since the infected cells do not synthesise the required artificial tRNA (Lund *et al.*, 1997).

3.4 INFECTION TARGETING

The receptor for the amphotropic (and GaLV) SU are expressed on many cell types and thus confer a promiscuous infection spectrum upon MLV-based

retroviral vectors. For reasons of safety and efficacy, it would often be desirable to redirect or limit the infection spectrum of retroviral vectors, so that only the correct target cell type is infected. This requires the modification or alteration of the MLV envelope proteins so that they interact with more exclusive cell receptors. A number of strategies have been employed to achieve this goal (reviewed in Salmons and Günzburg, 1993). In many of these studies, ecotropic MLV vectors were modified to demonstrate that infection targeting had indeed taken place since these retroviral vectors cannot normally infect human or other non-rodent cells.

Infection targeting has been attempted using antibodies, directed against known proteins that are expressed on the surface of the target cell, linked via streptavidin to antibodies specific for the virus Env protein. In this system antibodies directed against class I and class II major histocompatibility antigens (Roux *et al.*, 1989) or against the receptor for epidermal growth factor (Etienne-Julan *et al.*, 1992) gave targeted infection of cells expressing these molecules on their surface, but with low efficiencies. Another drawback with this, and indeed other, strategies is that not all receptors are competent for virus uptake or allow later steps in the viral life cycle to occur (Goud *et al.*, 1988).

In the past few years the most popular strategy for modification of the infection spectrum of retroviral vectors has involved the genetic engineering of the viral *env* gene carried in the retroviral packaging cell line. This has been facilitated by the identification of the regions of the SU Env protein involved in receptor recognition (Battini *et al.*, 1992, 1995; Morgan *et al.*, 1993; Ott and Rein, 1992). These regions have been replaced with gene segments encoding epitopes that would recognise other receptors such as erythropoietin (Kasahara *et al.*, 1994) or heregulin (Han *et al.*, 1995), thereby allowing the selective control of receptor targeting of the resultant chimeric Env protein. Alternatively, targeting ligands have been inserted at the amino terminus of the SU protein between amino acids 6 and 7 (Cosset *et al.*, 1995b; Marin *et al.*, 1996; Russell *et al.*, 1993). In these approaches, the ligand-Env protein is produced in the packaging cell line *in addition* to the normal non-modified retrovirus envelope protein, which is presumably required for stability since Env proteins are presented as trimers on the surface of the virus. The variable domain of single-chain antibodies specific for a defined receptor/cell surface protein have also been used to target retroviral vector infection (Chu and Dornburg, 1995; Marin *et al.*, 1996; Russell *et al.*, 1993; Somia *et al.*, 1995). Even though retargeting of the infection event by modification of the retroviral envelope protein has been successfully achieved by a number of groups, it is invariably associated with reduced titres, probably for similar reasons to those mentioned above. Such manipulations of the viral SU protein are also of limited use because of the relatively complex synthesis and processing of this protein, ensuring both its functionality and its ability to become incor-

porated into newly synthesised virus particles. These requirements place constraints on replacement or modification since the conformation of certain domains is likely to be critical. Clearly, more knowledge is required about the mechanisms that govern the normal functioning of retroviral envelope proteins before chimeric envelopes can be constructed that retain the ability of these proteins to recognise the receptor *and* initiate infection efficiently.

Recently, Steven Russell and colleagues (Nilson *et al.*, 1996) have described a new two-step strategy that promises to revolutionise targeting of retroviral vectors to predefined receptors. The viral envelope protein is modified by the linear addition of a protease cleavage site and the selected receptor ligand domain at the amino terminus of the SU protein. After binding the appropriate receptor on the target cell, the receptor/ligand can be cleaved off by expression of the protease (Nilson *et al.*, 1996). The retrovirus then attaches to its usual receptor on the same target cell, allowing the retrovirus to enter the cell by the natural route, circumventing the entry problems that often occur when retroviruses are targeted to use non-retroviral receptors (Etienne-Julan *et al.*, 1992). Titres of up to 10^6 cfu/ml (colony forming units per millilitre cell culture medium) have been achieved using the EGF binding domain as a means to target in this two-step system (Nilson *et al.*, 1996). The use of phage display libraries also promises to reveal ligands that will be useful for targeting purposes. Such libraries facilitate the rapid screening of peptides for their ability to bind to cell receptors present on specific cell types. Peptides identified using this screening system could be incorporated into any gene delivery system, including retroviral vectors, to achieve infection targeting (Barry *et al.*, 1996). The same system can also be utilised to achieve targeted infection by blocking retroviral infection of non-desired target cells. In this approach, the terminal end of the SU protein has been linked to a cleavage site, and in addition, to a peptide that masks the normal SU binding domain as well as to a specific ligand present on all non-target cells.

3.5 GENE EXPRESSION FROM RETROVIRAL VECTORS

3.5.1 GENERAL CONSIDERATIONS

The heterologous genes delivered by retroviral vectors can be expressed in a number of different ways (Figure 3.5). The retroviral promoter within the LTR can drive heterologous gene expression when such genes are cloned into the position formerly occupied by *gag*. Indeed, in many applications where it is advantageous to transfer and express two genes, for instance a therapeutic gene and a marker gene, the second gene can be inserted into the *env* position and expressed from the subgenomic viral RNA. Unfortunately the retroviral promoter is not particularly powerful and also suffers the disadvantage of

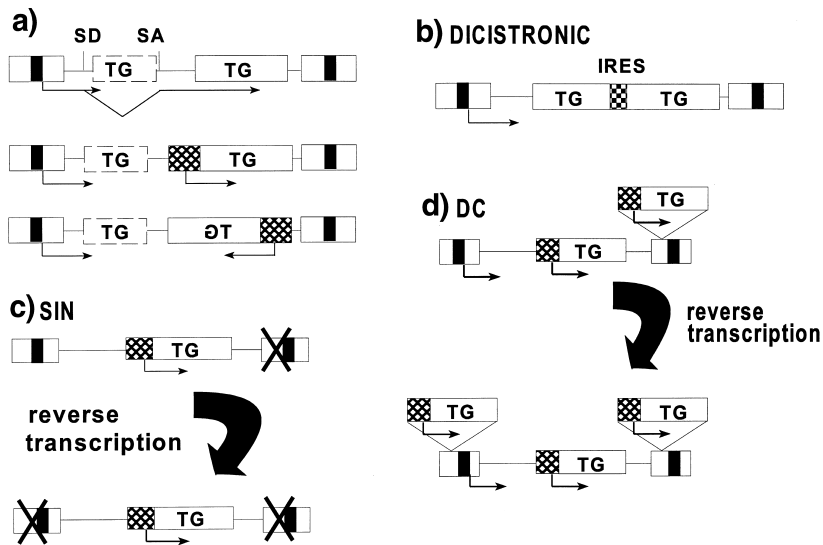


Figure 3.5. Expression configurations of retroviral vector constructs. Therapeutic genes (TG) can be expressed in a number of ways from the vector construct. In **(a)** and **(b)** the TG is expressed, either directly from the retroviral promoter as a genomic length transcript or (shown in **(a)**) as a spliced transcript. Retroviral vectors can accommodate one or two genes. The insertion of an internal ribosome entry site (IRES) allows a second gene to be expressed from the same genomic RNA, as shown in **(b)**. The TG can also be expressed from a heterologous promoter inserted into the retrovirus vector (hatched box in **(a)**), either in the sense or antisense orientation. Self-inactivating (SIN) and double-copy (DC) vectors as shown in **(c)** and **(d)** carry modified 3' LTRs that lead to either loss of the retroviral promoter (SIN) or a duplication of an expression cassette (heterologous promoter linked to the TG) in the infected cell.

being shut down or silenced after a variable period in many cell types (Lund *et al.*, 1996; Xu *et al.*, 1989) or in transgenic animals (Richards and Huber, 1993; Vernet and Cebrian, 1996). Methylation and host factors have been identified as culprits for this shut-off, and efforts have been made to alter retroviral LTR and downstream sequences to prevent transcriptional silencing in certain cell types (reviewed in Lund *et al.*, 1996).

Expression of genes delivered by retroviral vectors also depends on the site of integration in the host cell genome. Tissue-specific regulation from heterologous promoters (see below) may be overridden or compromised by strong cellular regulatory elements located in the vicinity of the integration site. Locus control regions (LCRs), regulatory elements that have been shown to confer position-independent, high-level expression upon genes (Dillon and Grosfeld, 1993), can be included in retroviral vectors to try to overcome

silencing problems. The inclusion of a 36 base pair LCR core sub-sequence from the human β -globin gene in a retroviral vector carrying a human β -globin gene was shown to enhance the expression of human β -globin in mouse erythroleukaemia cells, although expression levels were still therapeutically suboptimal (Chang *et al.*, 1992). However, a similar retroviral vector construct was shown to give widely varying levels of expression (4–146%) in different infected cell clones (Sadelain *et al.*, 1995). Thus, either more of the LCR region is necessary to obtain position-independent expression or LCR sequences are generally not able to function in the context of retroviral vectors. An alternative strategy to ensure position-independent expression of genes in retroviral vectors is to shield them from the effect of enhancers or repressors located in the vicinity of the integration site (Duch *et al.*, 1994). A number of such insulators have been identified in drosophila (Gerasimova and Corces, 1996) and mammalian cells (Felsenfeld *et al.*, 1996), and these could be incorporated into future retroviral vectors.

Self-inactivating (SIN) (Yu *et al.*, 1986) and double-copy (DC) vectors (Figure 3.5; Hantzopoulos *et al.*, 1989) utilise a unique feature of the retroviral life cycle, the reverse transcription of viral genomic RNA into a double-stranded form. During this process sequences from the 5' end of the RNA (U5) are duplicated and placed additionally at the 3' end of the DNA, while sequences from the 3' end of the RNA (U3) are copied onto the 5' end of the DNA. The process generates the identical LTR structures which flank the viral genome. The retroviral promoter is located within the U3 region, which means that the promoter that is used in the infected cell is derived from the U3 region at the 3' end of the viral RNA. In SIN vectors, this U3 has been deleted, leading to an integrated provirus that lacks a retroviral promoter in the infected cell. Inclusion of an internal heterologous promoter means that this will be the only promoter present to drive the expression of the delivered gene. In DC vectors, a cassette consisting of a heterologous promoter linked to the gene to be delivered is inserted in place of the U3 region in the 3' LTR of the vector, and becomes duplicated after reverse transcription, ensuring that the promoter–gene cassette is present twice (i.e. in double copy) in the target cell. The R region can also be used as a site for insertion of genes since cDNAs encoding MyoD and purine nucleoside phosphorylase (PNP) have been successfully inserted into this region of the LTR (Adam *et al.*, 1995).

The use of internal ribosome entry sites (IRES) in retroviral vectors allows two or more genes to be expressed from the same transcript expressed from a single promoter (Figure 3.5; Koo *et al.*, 1992; Levine *et al.*, 1991). These vectors are reported to give higher titre, permit the insertion of larger heterologous gene segments, and show more stable expression of transferred genes compared to two-gene, two-promoter vectors (see above), and may overcome the reported interference between multiple promoters present in the same retroviral vector (Li *et al.*, 1992; McLachlin *et al.*, 1993; Xu *et al.*, 1989).

3.5.2 EXPRESSION TARGETING

As well as infection targeting, through redirection of infection specificity (see above), it is possible to limit the expression of therapeutic genes using promoters from genes that are expressed specifically or preferentially in defined cell types. Thus, a retroviral vector may deliver a gene to many cell types (if infection targeting is not possible) but the gene will be expressed only in the required cell type. Strict specificity should combine both infection and expression targeting.

A plethora of promoters that are preferentially active in particular cell types have been utilised in retroviral vectors. These include the hepatocyte- (and hepatoma-) specific promoters from the phosphoenolpyruvate carboxylase (Hafenrichter *et al.*, 1994; Hatzoglou *et al.*, 1990; McGrane *et al.*, 1988), α -fetoprotein (Huber *et al.*, 1991), α_1 -antitrypsin (Hafenrichter *et al.*, 1994; Rettinger *et al.*, 1994) and albumin genes (Hafenrichter *et al.*, 1994) and promoters that are preferentially active in tumours such as from the tyrosinase gene for expression targeting to melanomas (Vile *et al.*, 1995) and *erb B-2* for mammary tumours (Harris *et al.*, 1994).

Early vectors expressed the delivered gene from tissue-specific or inducible heterologous promoters inserted into the body of the vector, i.e. in addition to the retroviral promoter. However, this configuration is often associated with transcriptional interference effects due to the presence of both the retroviral and the heterologous promoters. These interference effects can either be manifested as loss of expression from one or both promoters or as a loss of tissue specificity or inducibility of expression from the heterologous promoter. More recent vectors carry heterologous promoter/enhancer elements in the LTR in place of the retroviral promoter/enhancer (Ferrari *et al.*, 1995; Günzburg *et al.*, 1995; Salmons *et al.*, 1995; Vile *et al.*, 1995). Such vectors in which the promoter is converted from that of MLV in the packaging cell line to the introduced heterologous promoter in the infected target cell have been termed promoter conversion (ProCon) vectors (Figure 3.6; Günzburg *et al.*, 1995; Salmons *et al.*, 1995). It remains to be seen whether these vectors may be safer but (i) the lack of viral promoter sequences is expected to reduce the frequency of recombination with viral sequences in the producer or target cell and (ii) it has yet to be shown that a promoter from a cellular gene can activate or inactivate cellular genes in the context of a retrovirus.

3.5.3 INDUCIBLE PROMOTERS

The glucocorticoid inducible promoter of mouse mammary tumour virus (Günzburg and Salmons, 1992) has been successfully used in ProCon vectors to give regulatable gene expression in cell culture (Günzburg *et al.*, 1997;

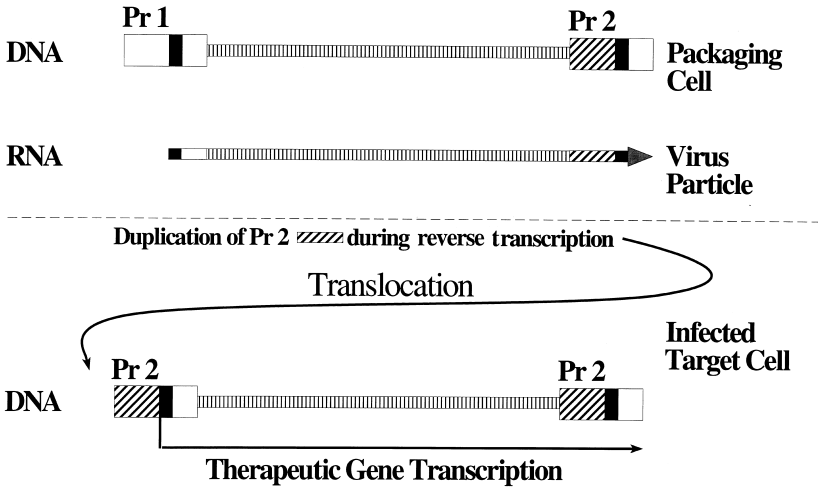


Figure 3.6. Retroviral vectors that undergo promoter conversion. The vector construct carries the retroviral promoter (Pr 1; promoter 1), in the U3 region of the 5' LTR, that directs expression of the viral genomic RNA in the packaging cell line. The 3' LTR promoter sequence of the virus has been replaced by the heterologous promoter chosen to drive gene expression in the infected cell (Pr 2). Note that this sequence (shaded) is the only promoter present in the genomic RNA that is packaged into the virus particle. Upon infection of the target cell, the RNA is converted to a double-stranded DNA molecule (as in Figure 3.1) by reverse transcriptase. This process results in the duplication of Pr 2 and the translocation of one copy to the 5' end of the proviral DNA, where it is the exclusive promoter used to drive gene expression in the infected cell.

Mrochen *et al.*, 1997). This promoter may be less useful as an inducible promoter *in vivo*, although it may enable the targeting of gene expression to the mammary gland and B lymphocytes (Günzburg and Salmons, 1992). Cannon and co-workers have used the ProCon principle to create an MLV-derived retroviral viral vector that uses the Tat inducible HIV promoter after infection of cells. This vector may be useful for targeting the expression of therapeutic genes to HIV-infected cells. Expression will be activated in and limited to these cells since only HIV-infected cells express Tat (Cannon *et al.*, 1996). Retroviral vectors carrying a tetracyclin inducible promoter to drive expression of genes have also been constructed (Paulus *et al.*, 1996).

3.6 RETROVIRAL VECTOR TITRES AND STABILITY

Virus and viral vector titres are usually measured by virtue of an effect that the virus has on target cells. Often in the case of viral vectors this is the

number of cells that receive and express an enzymatic marker gene enabling a colour reaction such as β -galactosidase, or alternatively an antibiotic resistance gene which allows infected cells to be identified on the basis of their ability to survive in antibiotic-containing medium. Thus, the titre represents functional units of virus and not the absolute number of virus particles. One commonly cited disadvantage of retroviral vectors is that the titres obtainable seem low when compared to other viral vectors such as adenovirus and adeno-associated virus vectors, even though there are reports of retroviral titres up to 10^7 cfu/ml. It has recently been shown that retroviral titres are actually much higher (up to one order of magnitude) than previously thought since culture medium containing vector virus can be shown to contain significant amounts of infectious vector virus when serially transferred to fresh, non-infected cells (Tavoloni, 1997). It is highly questionable whether functional titres determined on a limited number of established cell lines (such as NIH3T3 cells) in culture truly reflect the amount of vector virus capable of delivering a gene to primary cells or to cells *in vivo* (Forestell *et al.*, 1995). Further, it follows that optimisation of the virus titre on such established cells may be inappropriate since such cells do not necessarily reflect the biological properties of the relevant target cells for gene therapy. A number of physical and chemical methods have, however, been used to increase the apparent titre measured *in vitro*. This includes concentration (Kotani *et al.*, 1994; Paul *et al.*, 1993), flow-through infection, in which cells are grown on a porous filter and virus is passed over them (Chuck and Palsson, 1996), and treatment of vector-producing cells with sodium butyrate (Olsen and Sechelski, 1995; Pages *et al.*, 1995; Soneoka *et al.*, 1995). Considerable effort is also being put into gaining an understanding of the physico-chemical and biological factors affecting virus stability and consequently virus titre. The half-life of MLV retroviral vectors in cell culture medium has been measured to be somewhere in the order of 3.5–9 hours at 37 °C (Chuck *et al.*, 1996; Kaptein *et al.*, 1997; Paul *et al.*, 1993; Russel *et al.*, 1995; Sanes *et al.*, 1986; Tavoloni, 1997). Polycations such as polybrene (hexadimethrinebromide) and protamine are included in vector virus preparations to increase the efficiency of infection of cells. These positively charged molecules are thought to act as 'adaptor molecules' in alleviating the electrostatic repulsion between the negatively charged virus and negatively charged cell membrane (Figure 3.7; Coelen *et al.*, 1983; Hornsby and Salmons, 1994). Polybrene has, however, also been shown to affect the kinetics of retroviral decay and may cause aggregation of vector virus particles, thus preventing them from infecting cells (Andreadis and Palsson, 1997). Temperature also has an effect on virus titres. Surprisingly, production of retroviral vector virus at 32 °C results in better titres (Bunnell *et al.*, 1995; Kotani *et al.*, 1994). This has been shown to be due to a four-fold increase in the half-life of the recombinant virus at 32 °C as compared to 37 °C (Kaptein *et al.*, 1997).

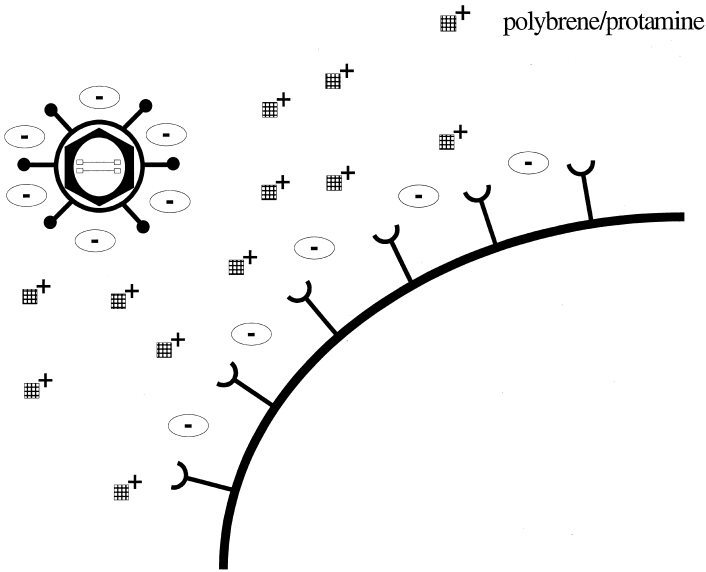


Figure 3.7. Polycations facilitate infection. The negatively charged virus and cell are shown. Polycations such as polybrene neutralise electrostatic repulsion between these two structures, thereby facilitating infection.

Titre is also dependent on the presence and abundance of cell-encoded receptor molecules on the surface of the target cell. The amphotropic receptor is expressed on many but not all cells. Non-dividing or slowly dividing hepatocytes express little amphotropic receptor, and haematopoietic stem cells may express less of this receptor than the receptor for GaLV (Bunnell *et al.*, 1995) (see also Section 3.3). In contrast, the receptor for another enveloped virus, vesicular stomatitis virus (VSV), is abundantly present on many different cell types (Coll, 1995). The VSV envelope (G) protein can be incorporated into retroviral particles, a process known as pseudotyping (Weiss, 1993), resulting in both increased titres as well as more stable virus particles than those carrying MLV envelope proteins (Burns *et al.*, 1993). These MLV/VSV pseudotyped particles additionally show a greatly expanded host range and cell type spectrum (reviewed in Friedmann and Yee, 1995).

Fast selection of successfully infected primary cells has not been practicable to date because the assays used for currently available marker genes often affect cell viability. Even the sorting of cells for the expression of β -galactosidase using the fluorescence-activated cell sorter (FACS) is stressful for cells due to cell permeabilisation required to allow the substrate to enter cells. Antibiotic resistance selection requires cultivation of cells for a number of days before non-transduced cells are killed off, although a quick

assay has now been developed for G418 (Byun *et al.*, 1996). Recently, a new marker gene has been used in retroviral vectors that finally allows facile selection in the FACS without any significant cell toxicity. This gene encodes the green fluorescent protein (GFP), which emits green light under ultraviolet (reviewed by Chalfie *et al.*, 1994). GFP genes have been engineered for better expression in mammalian cells, and these hGFP and EGFP genes have been inserted into retroviral vectors (Klein *et al.*, 1997; Muldoon *et al.*, 1997) and used to rapidly identify successfully infected cells.

3.7 LENTIVIRAL VECTORS

MLV-derived vectors, like MLV, are limited to infecting dividing cells because the pre-integration complex consisting of the viral DNA and integrase cannot be transported across the nuclear membrane (Lewis and Emerman, 1994; Roe *et al.*, 1993). During cell division, this membrane breaks down, allowing the viral DNA complex to reach, and integrate in, the genomic DNA of the target cell. In contrast, lentiviruses, such as HIV and simian immunodeficiency virus (SIV), are able to infect non-dividing cells (reviewed by Stevenson, 1996). This appears to be due to the presence of redundant nuclear localisation signals both in the HIV MA protein (Bukrinsky *et al.*, 1993) as well as in the HIV accessory gene product Vpr associated with the MA protein (Heinzinger *et al.*, 1994).

Due to this property of lentiviruses, attention has turned to the construction of lentivirus-based vector systems for gene transfer to quiescent and/or differentiated cells. At first glance, members of this subfamily of retroviruses, such as HIV, do not appear to be acceptable gene transfer vehicles because of their association with immunodeficiency. However, assuming that vector systems can be developed that are highly unlikely to lead to the production of replication-competent HIV and assuming that the viral components necessary to make vector particles are not themselves involved in causing the immunodeficiency, for example by stimulating an autoimmune response, these systems may be ideal for this purpose. A possible second advantage for creating vector systems based on lentiviruses is that there is no evidence to date for insertional mutagenesis by these viruses, although there is no known reason why this should not occur.

A number of groups have constructed vectors based upon HIV and, invariably, these systems have relied on the use of envelope proteins from other viruses and not from HIV, reducing the probability of recombination giving rise to infectious virus. The VSV G protein and the amphotropic Env of MLV have both been used in such pseudotyped lentiviral vectors.

It has been reported that HIV vectors pseudotyped with the envelope proteins of amphotropic MLV or VSV can give titres of up to 10^7 cfu/ml (Naldini

et al., 1996; Page *et al.*, 1990; Reiser *et al.*, 1996) or ~ 105 cfu/ml when pseudotyped with the envelope of human T-cell leukaemia virus (HTLV) (Landau *et al.*, 1991). The HIV accessory genes (such as *vpr*, *vpu* and *nef*) have been inactivated in the HIV vector system described by Reiser and co-workers and this is believed to contribute to the safety of such vectors (Reiser *et al.*, 1996). On the other hand, some of these gene products, for example Nef, have been postulated to enhance virion infectivity, and thus might be beneficial in the vector (Naldini *et al.*, 1996).

It has long been known that retroviruses (and thus retroviral vectors) are directly inactivated by human serum complement (Welsh *et al.*, 1975). It has been shown that complement-mediated inactivation is non-lytic and depends both on the retrovirus and on the cells from which the virus has been produced. For example, MLV produced from mouse cells is much more readily inactivated than MLV produced from human or mink cells (Takeuchi *et al.*, 1994). This is because human serum contains antibodies to the Gal(α 1-3)Gal modification of carbohydrates. Such modified carbohydrates are found on proteins of most mammals but not in humans since the enzyme that performs this modification ((α 1-3) galactosyltransferase) is not present in humans (Takeuchi *et al.*, 1996). Virus produced from non-human cell lines displays modified carbohydrates as part of its outer lipid bilayer, allowing it to be recognised by antibodies which recruit complement and cause inactivation by a non-lytic process. The use of established human cell lines, such as 293 (Pear *et al.*, 1993) or HT1080 (Cosset *et al.*, 1995a) for the construction of packaging cells has reduced this problem. Alternatively, the complement pathway can be inhibited by administration of monoclonal antibodies to specific complement components (Rother *et al.*, 1995). Lentiviral vectors based upon HIV would be less susceptible to complement – mediated inactivation than those derived from MLV.

3.8 CONCLUSIONS AND PERSPECTIVES

Retroviral vectors are the most commonly used gene delivery vehicles in clinical gene therapy trials and, as of June 1996, 969 patients have been treated with these vectors (Marcel and Grausz, 1996). Nevertheless, retroviral vectors have in the past few years lost ground, particularly to adenovirus vectors, due to their higher titres and infectivity spectrum. However, recent advances, some of which are mentioned in this review, have renewed interest in retroviral vectors. These advances have included (i) general design improvements, (ii) vectors based on lentiviruses, (iii) greater insight into retroviral stability and production, giving the hope of higher titres, and (iv) safer systems to ensure that replication-competent virus is not produced. The quest for the perfect retroviral vector is not yet over and is probably not

achievable. Synthetic vector systems will probably solve many of the problems associated with current gene delivery systems in general, but it is a safe bet that components of retroviruses will be included in the artificial vector systems of the future.

*NOTE ADDED IN PROOF

Climactic vector systems from which retroviral vectors are produced after delivery of the necessary components into target cells using other virus delivery systems (so-called "launching pad" systems) have been successfully developed based on adenoviruses, alphaviruses, herpes viruses and poxviruses (Reynolds, P.N., Feng, M., and Curiel, D.T. (1999) Chimeric viral vectors – the best of both worlds? *Mol. Med. Today* 5, 25–31.

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4 Lentiviral Vectors

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4.1 INTRODUCTION

Lentiviruses are a subfamily of retroviruses infecting both primate and non-primate hosts (for reviews on lentivirus biology and molecular biology, see [1]). They are characterised by tropism for cells of the monocyte/macrophage series. The non-primate viruses infect domestic animals, maedi visna virus (MVV) infecting sheep, caprine arthritis encephalitis virus (CAEV), infecting goats, and equine infectious anaemia virus (EIAV) infecting horses. More recently described are bovine immunodeficiency virus (BIV) and feline immunodeficiency virus (FIV) infecting cattle and cats, respectively. The primate lentiviruses human immunodeficiency virus types 1 and 2 (HIV-1, HIV-2) and simian immunodeficiency virus (SIV) infect humans and other simians, respectively, where they are, in addition, tropic for lymphocytes. HIV-1 and HIV-2 cause AIDS in humans. The closely related SIV is subdivided into different strains, which infect different primate species and which, according to the strain and the host, may cause anything from asymptomatic carriage through to an AIDS-like immunodeficiency syndrome. Although attempts are being made to develop a number of non-primate lentiviruses such as FIV and EIAV as retroviral vectors, as yet there is little progress with viruses other than HIV-1, HIV-2 and SIV. This chapter describes current knowledge of vectors based on HIV with details on SIV and viral chimeras where appropriate.

The retroviral life cycle is illustrated in Figure 4.1 [2]. Lentiviruses are complex retroviruses with multiple spliced RNA species and a number of small regulatory proteins that allow sophisticated control of the viral replicative process with a distinguishable early and late phase to their replicative life cycle [3,4].

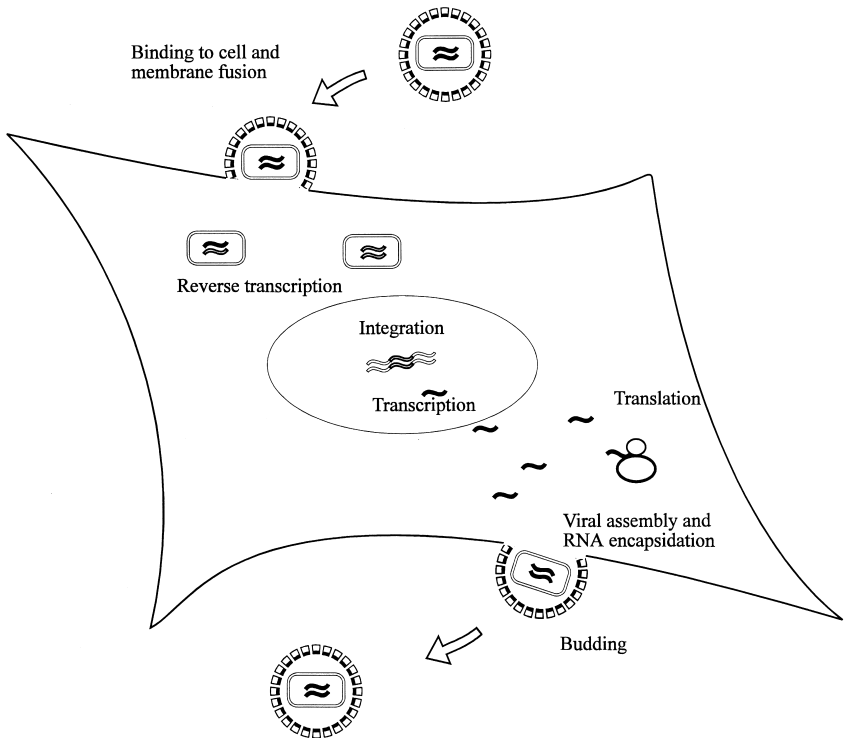


Figure 4.1. typical retroviral life cycle demonstrating infection of cell, conversion of genomic RNA to double-stranded DNA and integration into chromosome of cell, followed by transcription, viral protein production assembly encapsidation and budding of virus.

4.2 GENETIC STRUCTURE OF LENTIVIRUSES

The gene complement and RNA splicing pattern of HIV-1 as the lentiviral prototype are shown in Figures 4.2 and 4.3. In the provirus, the 5' and 3' ends are identical long terminal repeat (LTR), non-coding regions containing important *cis*-acting sequences. There are three major open reading frames: *gag*, coding for the viral capsid proteins, *pol*, coding for the virus enzymes, and *env*, coding for the envelope glycoproteins. Using HIV as the prototype, the *gag* and *pol* genes are encoded on the unspliced viral message. The Gag polyprotein is initially synthesised as a Pr55 precursor, which is then cleaved by the virus's own protease into at least four component segments. This cleavage occurs during or after budding. The most N terminal fragment is the matrix protein (MA) which, in the virus particle, occupies a peripheral site

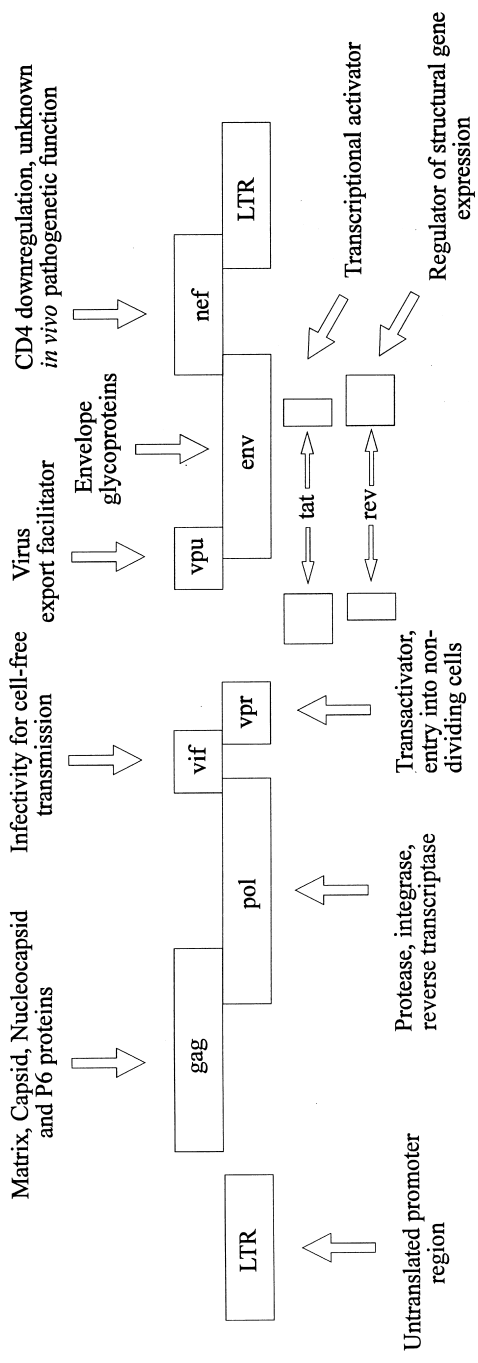
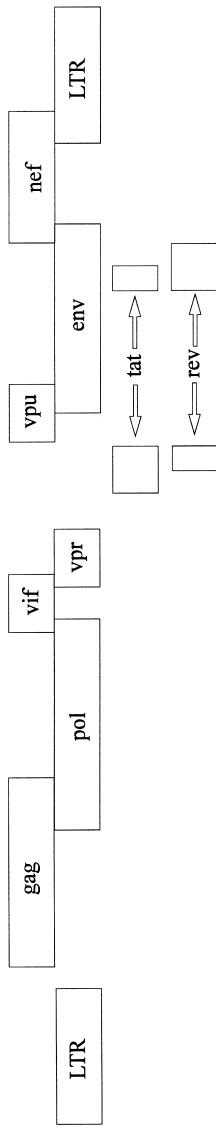


Figure 4.2. Gene complement of HIV as prototype lentivirus showing major open reading frames and function of gene products.



mRNA species

Unspliced

gag, pol

Singly spliced

env, nef

Multiply spliced

tat, rev, etc.

Figure 4.3. Schematic of the family of mRNA molecules derived from the provirus, falling into three groups: unspliced, singly spliced and multiply spliced.

inside the envelope and is involved in anchoring the envelope proteins into the virion [5,6]. Following virus entry into the cell, MA also appears to be involved in targeting the integration complex to the nucleus and permitting integration of the provirus into a non-dividing cell [7]. A putative nuclear localisation signal has been identified within its amino acid sequence which may be responsible for this. C terminal to MA is the capsid (CA), which is the major structural protein of the virus core. It is also the protein recognised in some commercial ELISA assays for virus particles (p24 in HIV-1). It is responsible for the structural integrity of the virus core [8]. The p9 or nucleocapsid (NC) protein is the third segment of *gag*. This has two zinc finger-like CCHC motifs and is involved in coating the genomic RNA [9] within the virus to protect it from degradation. It also has functions after viral entry facilitating the reverse transcription of the viral RNA [10]. Recent work suggests that the 'spacer' peptide P2 between CA and NC may influence RNA encapsidation [11]. The most C terminal fragment of *gag* is p6, mutation of which leads to a defect in the terminal events of viral budding from the cell [12,13]. The exact mechanism by which p6 influences the final stages of virus assembly and budding is unclear although it may involve interactions with cellular proteins.

The *pol* gene products are produced initially as a Gag/Pol fusion protein. In the case of HIV this occurs during translation where a -1 frameshift occurs towards the end of the *gag* open reading frame, resulting in the ribosome beginning to translate in the *pol* open reading frame [14]. The fusion protein produced is a Pr160 Gag/Pol precursor. The *pol* gene products include, from the *N* terminal, protease (PR), which cleaves the Gag and Gag/Pol proteins into their component parts, reverse transcriptase (RT), responsible for converting the RNA genome into a double-stranded DNA molecule, and integrase (INT), which is then able to insert the double-stranded provirus into the cellular chromatin, dependent on specific recognition sequences in the viral LTRs.

Env protein is translated from a singly spliced RNA. The major splice donor is in the untranslated region 5' of *gag*, and the *env* splice acceptor is upstream of the *env* initiation codon. A singly spliced transcript also codes for Nef. The Env protein is translated to produce a gp160 precursor on endoplasmic reticulum (ER) bound ribosomes. Gp160 is translocated into the ER lumen, cleaved by cellular proteases into a transmembrane gp41 (TM) and a surface gp120 (SU) protein. These associate non-covalently, undergo complex glycosylation and are exported to the cell surface, probably as a trimeric or possibly a tetrameric complex.

In addition, HIV has a number of small open reading frames for which there are homologues in most lentiviruses [15]. Tat is a powerful transactivator of transcription encoded on a doubly spliced message which acts in

trans to enhance processivity of RNA polymerase through an RNA stem loop structure formed in the region downstream of the transcriptional start site in the LTR. This is known as the Tat-responsive (TAR) stem loop [16]. The Tat/LTR combination is one of the most powerful promoters known and enhances production of all viral mRNA species. In early infection due to instability sequences (INS) found within the *env* intron (i.e. the *gag* and *pol* sequence) and in the *env* gene itself, full-length messenger RNA is rapidly processed to doubly spliced forms [17]. Tat is thus the major gene product early in the life cycle and feeds back positively, enhancing its own synthesis.

The Rev protein is encoded by two exons which overlap those of Tat. This protein is also produced in abundance early in the life cycle and as it accumulates it is able to interact with a Rev-responsive element (RRE) in the *env* coding region to facilitate export of singly spliced and unspliced messenger RNAs from the nucleus to the cytoplasm. The Rev/RRE interaction appears to overcome the effect of the INS, which favours splicing. This gives a temporal control to viral RNA expression, in which large amounts of RNA coding for regulatory proteins are produced in the early phase followed by a Rev-mediated switch to production of singly spliced and full-length messenger RNAs coding for the structural and enzymatic proteins of the virus. Four other small open reading frames merit mention. The *nef* gene situated at the 3' end of the virus has a number of functions. Although initially identified as a negative regulator of transcription [18], it appears to have other more important functions *in vivo*, including downregulation of CD4 [19] from the infected cell surface and as yet unidentified essential functions which lead to small mutations in *nef* (in SIV) being repaired by the virus on passage *in vivo* yet reappearing on passage *in vitro* [20].

Vif is an accessory protein needed in some systems for cell-free infection. Cell to cell transmission of virus occurs efficiently in Vif positive or negative viruses but in a number of cell lines, cell-free infection can only occur if Vif is present [18]. Recent evidence suggests Vif is packaged into the virus particle and may affect an early step in virus uncoating [21].

Vpu has the function of increasing the efficiency of viral export. This appears to have two components. First, it disaggregates complexes between the envelope protein and the CD4 protein of an infected cell, which occur in the endoplasmic reticulum during export of both proteins, allowing envelope export. Second, it has a more generalised export function evidenced by the inability of HeLa cells to export viruses or envelope-free Gag particles in the absence of Vpu [22,23,18].

Vpr is a protein with a number of suggested and identified functions [24]. It is incorporated into the virus particle [25] (unlike Vpu). It has a transactivating capability which is weaker than Tat [18]. It appears to contribute to the ability of HIV to integrate its genome into a non-dividing cell [26]. It has been shown to induce cell cycle arrest in G2 in some cell lines

[27]. Thus, from a vector perspective, it has both desirable and undesirable properties.

4.2.1 ADVANTAGES OF LENTIVIRAL VECTORS

Like all retroviral vectors, lentiviruses offer the potential for stable single-copy gene insertion into a host cell chromosome and targeted gene transfer into cells bearing an appropriate viral receptor. The major reason for considering lentiviruses over other retroviruses is their ability to target cells that are in G_0 [28,29]. Many of the cell populations to which therapeutic gene delivery is targeted, such as liver, muscle and brain, spend most or all of their time in G_0 .

The second advantage is the feasibility of high-level gene expression using the powerful Tat/LTR promoter combination.

Thirdly, the accessory genes provide additional regulatory functions such that genes can be made inducible.

4.2.2 DISADVANTAGES OF LENTIVIRAL VECTORS

The obvious disadvantage of an HIV-based vector lies in the danger of recombination producing a wild-type virus. This can only occur if all the components necessary to re-create a complete viral genome are present, and the system should ideally be designed so that this cannot happen. A second disadvantage encountered to date is the relatively low titre of lentiviral vector systems. It is not yet clear why this is so. However, the perception of the problem is somewhat artificially exaggerated by direct comparisons of lentiviral vectors with murine vectors on transformed (often murine) cell lines *in vitro*. In general, researchers have found that when assessed for their transducing ability for primary cells *in vitro*, the difference between lentiviruses and murine-based vectors is much less marked, i.e. the murine vectors are less efficient as well.

4.3 LENTIVIRUS-BASED VECTORS

A retroviral vector system has two fundamental components [30]: (i) a modified viral genome containing the foreign gene of interest with *cis*-acting sequences necessary for its encapsidation into the virus particle and stable insertion into target cells – the vector – and (ii) a helper virus or viral construct(s) providing in *trans* all the structural and enzymatic proteins necessary to create an intact virion into which the vector can be packaged for delivery.

The minimal components for a vector are: packaging signal(s), which allows selective encapsidation of viral RNA into a particle, a primer binding

site (PBS) for initiation of reverse transcription, a polypurine tract (PPT) for initiation of proviral plus strand synthesis, and LTR sequences which supply both promoter and enhancer functions, polyadenylation signals and the recognition sequences required for provirus integration. All of these can be omitted from the packaging system constructs. In lentiviruses, the 5' LTR and primer binding site are almost contiguous and are usually included as one continuous sequence in the vector. The position of the encapsidation signal ψ in HIV is still a matter of some debate. Initial studies identified a sequence important for encapsidation between the 5' splice donor and the *gag* initiation codon [31–33]. Mutations in this region led to a variable packaging defect ranging from 10- to 1000-fold less than the wild-type virus partly dependent on the cell type. Subsequent studies have identified different additional regions as apparently able to enhance packaging, but with each study performed in a different system, drawing conclusions is difficult. The 3' end of the *env* gene, including the RRE (but not dependent on it), appears to enhance packaging of some constructs [34,35]. The 5' region of the *gag* gene has been identified as containing a packaging enhancing signal [36]. The TAR structure has also been suggested as being an important component of the packaging signal [37]. The packaging signal has been suggested to be discontinuous and made up of several different motifs [38]. The minimum sequence or sequences required for packaging have not yet been identified. However, under some circumstances, the 3' end of *env*, all of *gag* and the TAR region are dispensible for packaging whereas there is still general agreement that the region between splice donor and *gag* ATG has the most influence. A pentanucleotide sequence GNGR appears to be a commonly occurring motif in packaging signal regions of primate lentiviruses [39]. In practical terms, many vectors include the complete 5' region from the transcription start site through to the beginning or into the first part of the *gag* gene together with the 3' end of *env*. The latter is included either on the basis of it being a separate packaging enhancing signal or to overcome the instability sequences present on incorporating the 5' end of the *gag* gene into the construct (see Figure 4.6).

4.3.1 OTHER INFLUENCES ON PACKAGING

RNA packaging in HIV appears to be more promiscuous and less specific than in murine and avian retroviral systems. Several publications attest to the ability of HIV to encapsidate spliced RNA, either virus derived or from vectors, at a reasonably high efficiency although lower than that of the full-length unspliced message [40,41]. It is not clear whether this results from heterodimer formation between spliced and full-length species. A number of factors appear to influence this. In certain cell types used for transient transfection, such as Cos cells, there appears to be significant packaging of

the spliced RNA, possibly as a result of an extremely high cellular concentration of this species. In T lymphocytes the packaging is more specific although spliced RNA can still be detected within the virion particles. No cell line has been shown to be able to discriminate completely full-length RNA for encapsidation when transfection techniques have been employed. It is possible that high-level expression, which is the aim of transfection, over a short period of time, leads to a saturation of the system through the sheer quantity of RNA available in the cell. In experiments designed to identify packaging signals, *infection* procedures have been associated with a higher specificity of packaging than transfection [42]. Concurrent with this has been the observation that the packaging defect associated with small deletions in the 5' leader is much greater in infection-based experiments. In HIV-2 we have demonstrated a hierarchy of packaging specificity in which packaging in Cos cell transfections is less specific than in T cell transfections, which is again less specific than in T cell infections [42].

Even in packaging cell lines based on HIV, there is evidence that spliced RNA derived from a stably integrated proviral construct may still enter the packaging pathway and be incorporated into the virions. If this is the situation *in vivo* it would at first appear distinctly disadvantageous for the virus in that spliced RNAs would compete for packaging with the desired full-length genome. However, a small degree of promiscuity would also have the effect of enhancing heterodimer encapsidation, recombination, and with it the possibility for increasing viral diversity. It is also possible that the system is being assessed under artificial conditions and that the specificity may depend largely on the qualitative difference in RNA available for encapsidation when the viral capsids are assembling. Lentiviruses undergo a switch from early to late gene expression, and it may be this coincidence of increased unspliced RNA appearing in the cytoplasm together with structural proteins being produced which tips the balance against packaging of multiply spliced RNAs, which are more abundant earlier in the life cycle.

In practical terms, there is as yet no satisfactory cell line which will eliminate encapsidation of spliced RNAs. Those based on lymphocytes appear in our hands to be somewhat better than those based on epithelial or fibroblast-like cell lines. Sufficient cell lines have not yet been studied to be able to give categorical recommendations.

A further twist to this comes from the recent observation that HIV-1 can package HIV-1 or HIV-2 based vectors but that HIV-2 can only package HIV-2 vectors [11]. This appears to be due to a preference by HIV-2 for packaging RNA co-translationally, thus limiting its ability to pick up heterologous vector RNA in *trans*. The ability of HIV-1 to package vectors may be due to its decreased dependence on packaging in *cis*. Hence, its ability to act as a packaging system for other RNAs may depend on this promiscuity of encapsidation (Kaye and Lever, personal observations).

Certain heterologous genes appear to be associated with an unexplained difficulty in packaging under some conditions. The chloramphenicol acetyl transferase (CAT) gene appears to be encapsidated extremely well into a virion particle when it is included within the genome as a substitute for the *nef* [11] gene or in the middle of a deleted envelope gene. Inclusion of the CAT gene within the *gag* region [34] appears to have a negative effect on encapsidation, possibly due to the proximity of the heterologous sequence to the packaging signal where it might interfere with the secondary structure of the packaging signal.

The aim of a vector system is to deliver a heterologous gene to a target cell line. A variety of methods involving HIV have been tried. These range from gene substitution in a replication-competent helper virus through to conventional separated constructs designed to minimise or abolish the possibility of generating replication-competent virus. The major gene transfer papers using lentiviruses with native virus envelopes are summarised in Table 4.1.

4.3.2 DIRECT GENE TRANSFER

The first demonstration of this was performed by substituting a heterologous gene (CAT) for the *nef* open reading frame of a full-length HIV (Figure 4.4). A series [43] of CAT constructs of different lengths were inserted into the 3' end of the virus and some were found not to affect replication competence, delivering the full-length genome with the CAT gene intact to target cells, the CAT gene most likely being expressed from a spliced transcript. *In vitro*, the replication competence of the virus decreased as the size of the insert increased. This was postulated to be due to the increased size of the full-length transcript imposing an extra packaging constraint. Although there is evidence that greater than genome length RNA is packageable (McCann and Lever, personal observations), the maximum size of an RNA that can 'fit' into a lentivirus has never in fact been formally analysed. With the smallest CAT gene insertion, the competence of the virus to replicate *in vitro* was in fact slightly enhanced over the wild-type, probably because *nef*, which had been deleted, itself has a minor inhibitory effect on virus replication *in vitro*. Thus, for delivery of marker genes this is probably the most efficient system. However, none of the virulence of HIV is attenuated.

4.3.3 HELPER VIRUS MEDIATED

In earlier packaging studies using HIV where the exact *cis*-acting sequences were still being analysed, stable expression of test vectors in a CD4 positive cell line was used [34]. These cells were infected with wild-type HIV and the supernatant virus from these cultures was analysed by RNA slot blot and also by gene delivery to a second population of CD4 positive cells to calculate

the vector titre (Figure 4.5). Using such a system, gene transfer of vectors with titres of 10^3 and 10^4 cfu/ml (colony forming units per millilitre) was achieved. Because of the nature of this system, wild-type helper virus was present.

4.3.4 CO-TRANSFECTION SYSTEMS

4.3.4.1 Envelope Complementation

A simple vector methodology employed by a number of groups is that of *env* complementation. In this, a heterologous gene is introduced into the central portion of a deleted *env* open reading frame (Figure 4.4). The open reading frames for the accessory proteins Tat and Rev are left intact. The heterologous gene introduced is a marker gene such as CAT or β -galactosidase [44]. This plasmid is then co-transfected into a cell together with an envelope expressing gene and between them they produce viral particles in which the full-length RNA containing the heterologous gene in the envelope region is encapsidated. This has particular strengths when studying the envelope protein itself and has been used extensively for functional analysis of envelope mutants, where it has given a quantitative read-out of virus entry into a target cell population [45]. It also has applications in pathogenesis studies in that the system has single-round replication kinetics and can deliver a suitably detectable heterologous gene. It is able to provide information on subjects such as the first cell population susceptible to viral infection through various different routes of infection. The large degree of overlap between the envelope gene sequences in the two constructs raises the spectre of recombination and regeneration of full-length wild-type virus. This system, therefore, is not applicable for *in vivo* use in humans. However, in animal models using other lentiviruses, such as visna and SIV, it is valuable for studies of infectivity and pathogenesis.

4.3.4.2 Co-transfection Using Independent Vector and Packaging Constructs

Several groups have published data describing the practicability and efficiency of this system [46–48]. The number of separate DNA constructs co-transfected into cells is either two (packaging construct plus vector) or three (two complementary packaging constructs plus vector) (Figure 4.6). In the first case, systems have been designed to minimise commonality between sequences in the vector and the packaging construct by removal from the latter of LTRs and by deletion or mutation of the 5' leader packaging signal sequence, leaving intact only the open reading frames and appropriate splicing signals. Using this system, helper-free transduction of CD4 positive cells was demonstrated with titres of less than 10^2 cfu/ml.

Table 4.1 Summary of major gene transfer papers using lentiviral-based vectors

Reference	Co-transfection/ infection/stable cell line	Particle producing cells	Target cell	Packaging construct	Vector	Titre (cfu/ml)	Comments
[43]	Transfection	Jurkat	Jurkat		WT Gene in place of nef	> WT	Heterologous gene in place of nef in replication- competent virus Envelope complementation
[44]	Co	Cos-7	HeLa T4	FL Δ env and env expressor	Gene in env ORF	Up to 3×10^5	
[49]	Co	Cos-1	Jurkat	(1) FL $\Delta \Psi$ SV40pA (2) FL Δ (l env SV40pA and env expressor	T – R –	Up to 10^5	Highest titre published. Later publication from same group suggested this was difficult to reproduce
[47]	Co	Cos-1	HeLa T4 CEM	FL $\Delta \Psi$ CMV promoter SV40pA	T – R –	< 10^2	Titre equivalent to amphotropic Moloney virus
[62]	Co	Cos-1	HPB-ALL HeLa HeLa T4	FL Δ env and murine amphotropic env expressor	T – R –	10^2	722 nt at ' end of gag enhanced packaging

[63]	Co	Cos M6	HeLa T4	FL CMV promoter SV40pA CMV promoter $\Delta\Psi$ Δ env SV40pA and env expressor	T – R – HIV and SIV Various T –	HIV < 500 SIV < 50 10 ²	Cross-packaging of HIV and SIV RRE dispensible 653 ntT at 5' end of gag enhanced transfer, RRE dependent. Otherwise RRE dispensible High titres with Cos defective helper but with recombinant virus. No helper virus generated with two-plasmid packaging system – CD4 specific 3' end of env enhanced packaging Genes in gag region inhibit packaging CD4 specific Evidence for poor gag processing TET inducible system
[48]	Co	Cos-1	Jurkat				
[34]	Co	Cos-1	HeLa T4 HeLa T8 Jurkat-tat	WT WT $\Delta\Psi$ WT $\Delta\Psi$ Δ env and env expressor	T + R + T – R – Various	Up to 24 < 50	
[46]	Stable vector line WT virus infection	Jurkat (\pm Tat)	HeLa T4 Jurkat (\pm Tat)	WT	Various	Up to 10 ⁴	
[50]	Stable producer cell line	Vero	SupT1	FL $\Delta\Psi$ Δ 3'LTR	T – R –	10 ²	
[51]	Stable packaging cell line	HeLa T4	FL $\Delta\Psi$	Δ env Δ 3'LTR tet rev + env	T – R – T + R + (FL Δ env)	10 ³ 10 ⁴	5 + days to induce

Table 4.1 (*cont.*)

Reference	Co-transfection/ infection/stable cell line	Particle producing cells	Target cell	Packaging construct	Vector	Titre (cfu/ml)	Comments
[53]	Stable packaging cell line	CMT3-Cos	HeLa T4	CMV promoter $\Delta\Psi$ gag pol and either CMV promoter rev CMV promoter env or CMV promoter env CTE	T – R –	Up to 10^4	Nef enhances titre
[52]	Stable packaging cell line or incomplete producer line	SW480 MDS	HeLa T4	$\Delta\Psi\Delta$ env Δ 3' LTR and LTR env or $\Delta\Psi\Delta$ env Δ 3' LTR and vector	T + R + LTR or T – R –	10^2	THP-1 and HeLa Not useful as packaging cells

Co, co-transfection; FL, full length virus; Δ env, deletion in envelope gene; $\Delta\Psi$, deletion in 5' leader packaging signal sequence; SV40pA, polyadenylation signal from SV40; WT, wild type virus; 3' LTR, deleted 3' long terminal repeat; tet, tetracycline repressor based inducible system; CTE, Mason Pfizer monkey virus constitutive transport element; T, tat (T –, absent tat); R, rev (R –, absent rev); RRE, Rev-responsive element.

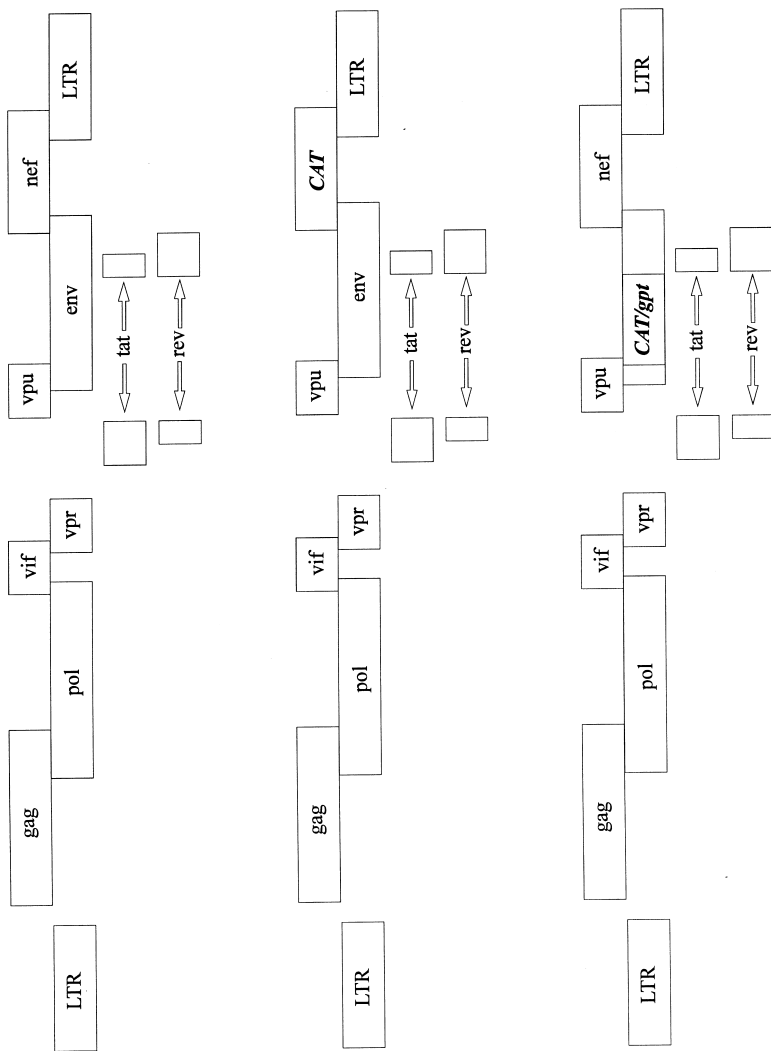


Figure 4.4. Alternatives for insertion of heterologous genes into a proviral construct. The upper panel shows the intact provirus, and the central panel demonstrates insertion of *CAT* gene in place of *nef*. This is replication competent. The third panel shows *CAT* or *gpt* inserted in place of the envelope gene. This requires complementation with an envelope expressor.

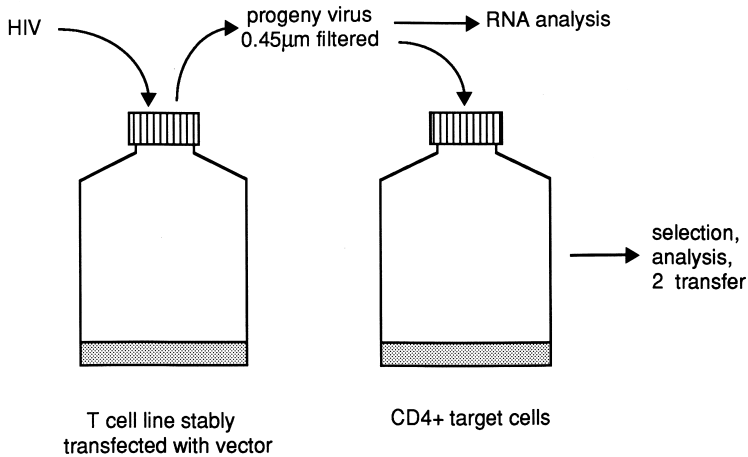


Figure 4.5. Helper virus mediated gene transfer. Useful for studies of encapsidation.

Importantly, this was shown to be no worse than a murine vector-based system assessed in parallel (personal observations). Where two-plasmid co-transfection has been used with extensive overlap between vector and helper virus, replication-competent virus is almost inevitably generated, even when packaging signal sequences have been deleted from the helper virus. Even a deletion of 35 base pairs in the packaging construct/helper, which has a major inhibitory effect on packaging in T cells, was insufficient to prevent recombination [46]. The weakness of co-transfection systems are that they are, for reasons of efficiency of expression, used in cells that have been shown in packaging studies to be relatively promiscuous and will package, at reasonable efficiency, RNA with deletions in otherwise important *cis*-acting packaging sequences. Recombination between heterodimers during reverse transcription after infection of the target cell is probably the source of replication competent virus.

4.3.4.3 Three-plasmid Co-transfections

Transient transfection assays using three plasmids have to date been found not to generate replication-competent virus, probably because this would require at least two recombination events between three constructs. The envelope construct is commonly driven from a heterologous promoter or a deleted LTR and has a heterologous polyadenylation signal. To date, both the HIV LTR and heterologous promoters have been used to generate the Gag/Pol proteins. The envelope expressor has usually had a heterologous promoter. The maximum titre so far documented for co-transfection delivery is in

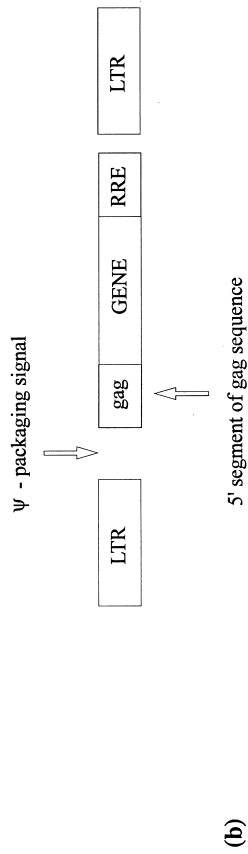
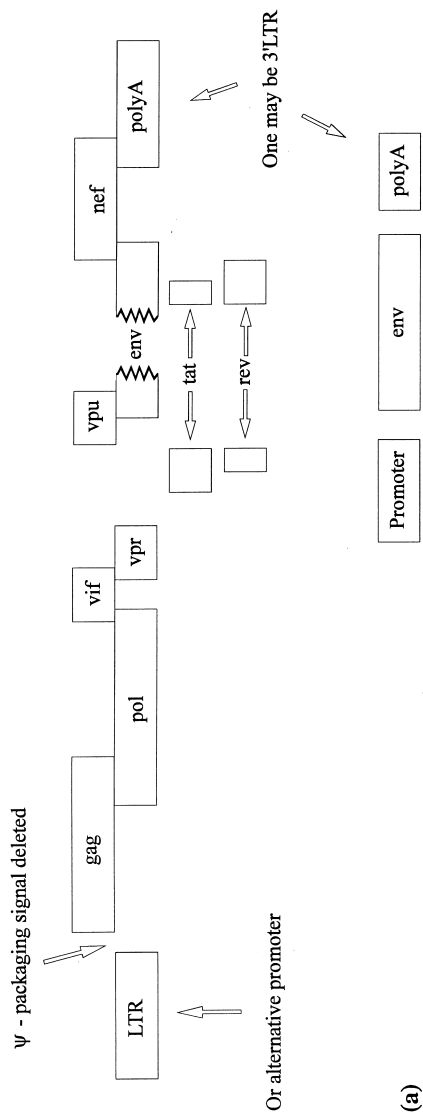


Figure 4.6. (a) Commonly used complementary constructs including all of the retroviral proteins. (b) Typical structure of HIV-based vector, where gene represents a heterologous gene which may be under control of its own promoter or, if in antisense, under the control of its own promoter and polyadenylation signal.

the order of 10^3 cfu/ml. The majority of publications suggest a lower titre of around 10^2 cfu/ml. An early paper suggesting extremely high efficiencies of 10^5 cfu/ml has not been replicated [49]. The system depends critically on having cells that are adept at taking up transfected DNA and that are tolerant of relatively large concentrations of transfected DNA, such as Cos-1. In theory there is no reason why cell lines stably expressing any one of the three plasmids should not be used to reduce the number of DNA constructs introduced at any one time. For example, stable expression of a vector containing a selectable marker in a cell line would ensure that every cell in the population that was transfected was expressing that vector so packaging constructs encoding only for the envelope and for the *gag/pol* regions would need to be introduced. There is a broad area of overlap between this and the next section on packaging cell lines but a general principle is that stable expression of the construct encoding the proteins that are most toxic to the packaging cell line might best be avoided and instead introduced by transient transfection into the system.

4.3.5 PACKAGING CELL LINES

For HIV there are now several publications [50–53] documenting packaging cell lines stably expressing structural proteins of the virus. In general these have been modelled on murine systems in which the envelope gene is expressed separately from the *gag/pol* construct. Ideally, these are generated using sequential steps in which limiting dilution of cells expressing one construct is performed. High-level expressors of that particular construct are then cloned out and used for the introduction of the next expressor and, again, limiting dilution is performed and a clonal population of high-level expression is selected. The very striking difference in the ability of different clones to express viral proteins justifies this approach and, for example, differences in p24 expression of 10^2 – 10^3 -fold can be found in different clones derived from HIV *gag* expressing lines [52].

Most groups have created a packaging cell line in which the *gag/pol* construct is transfected into cells with the help of a stable selectable marker. Expression of *gag* is Rev-dependent, thus the *gag/pol* expressor must also include the RRE. This is a potential weakness in that the envelope expressor also will need to contain the RRE. Many groups also choose to include the same sequence in their vectors to optimise expression leading to all three of the constructs involved in production of infectious vectors containing the RRE, and this raises the risk of recombination. Point mutations have been introduced into the *gag* gene by Pavlakis's group [54] such that the nucleic acid sequence is changed but the amino acid coding sequence is unchanged. This apparently removes the *cis*-acting INS effect and it is claimed that *gag* is produced in a Rev-independent manner. This would be one possibility for

avoiding inclusion of the RRE. An alternative strategy would be to include another *cis*-acting sequence which could substitute for the RRE. In Mason Pfizer monkey virus (MPMV) a *cis*-acting sequence – the constitutive transporter element (CTE) – has been shown to be able to act independently of Rev, to allow RNA export from the nucleus [55]. Thus, a *gag/pol* expressor could include the CTE rather than the RRE. The envelope expressor already by definition includes the RRE as it is part of the *env* sequence. Recent work suggests that for the vector the RRE leads to greater efficiency of transfer than the CTE [56].

The genetic complexity of HIV-1 means that not only *env* and *gag/pol* genes need to be expressed but also the regulatory genes *tat* and *rev*. The accessory genes *vpr*, *vpu*, *vif* and *nef* are dispensable for vector transfer into T cells when Cos-1 cells are used for virus production but may be required in other circumstances. Vif, for example, is absolutely required for particle infectivity when the virus-producing cell is restrictive for Vif defective viruses. The Vpu protein considerably enhances the release of virus particles from various cell types and Vpr may be required for integration into G₀ cells. The HIV genome can be split into 5' *gag/pol*, *vif* and 3' *tat*, *rev*, *vpu* and *env* coding fragments. However, balanced expression of all these latter genes from the 3' genome fragment may be problematic when they are placed outside the genetic context of the provirus. In addition, if one is beginning by selecting for a *gag/pol* expressor, Rev needs to be present in the system.

4.3.5.1 Problem Proteins

Lentiviral proteins pose a number of unique problems as some of these have proven difficult to express stably in certain cells. The viral protease enzyme, which is essential for processing of the Gag and Pol precursor proteins, appears to be toxic in some cell lines [57]. This may be due to the protease being able to cleave certain cytoskeletal proteins. Cell lines expressing protease were found only to grow when point mutants arose in the protease gene, rendering the enzyme defective. Other groups have found, usually using human-derived cell lines, that longer term protease expression is possible.

The *env* gene product is also potentially toxic, particularly to cells that are CD4 positive. A number of cell lines are described as being CD4 negative yet in some cases have detectable CD4 messenger RNA. It is probable that the level of CD4 on their surface is below the limit of detection by conventional histological staining techniques. In other cases, cells might be expressing receptors involved in CD4-independent viral entry. However, any expression of CD4 in a cell population also expressing gp120 can lead to cell fusion and death. Unfortunately, some of the best HIV particle producing cell lines are lymphocyte-derived and have CD4 on the surface. Again, in principle, it

is preferable to have a packaging cell line that does not have CD4 on the surface as this minimises the chances of re-infection of the cell line by the vectors that are being produced, a process which increases the likelihood of recombination.

The Vpr protein, important in entry into G_0 cells, may also be problematic. In a number of cell lines Vpr has been shown to induce cell cycle arrest. It has been shown to be necessary for Vpr to be produced in the cell line from which the virion is produced so that it can be encapsidated [18]. Experiments have clearly shown that providing Vpr in *trans* in the target cell line does not work. In a three-plasmid system (*gag/pol*, *env*, vector), the most practical approach is to have two of these constructs stably expressed and introduce the third one by transient transfection with the *vpr* open reading frame being included in this latter construct. Vpr has a degree of species specificity such that, for example, HIV-1 Vpr may have its greatest effect in human cell lines and SIV Vpr in simian cell lines, both having less effect in heterologous cells. This offers scope for using heterologous vectors to avoid growth arrest. A common observation in lentivirus packaging cell lines stably expressing viral proteins is a progressive decline in viral protein and, hence, viral particle production. The mechanism for this is not clear.

4.3.5.2 Inducible Constructs

To overcome some of these problems, a packaging cell line has been described in which components are inducible [51]. This was achieved using the powerful tetracycline repressor system (tet) to control Rev and Env products. This allows for short-term high-level expression of the desired proteins with genetic shut-off in between. The kinetics of production of structural protein in this system were unusually slow for a tet-based system, with induction taking over five days to maximise despite, in some cases, the presence of *rev* in *trans* from the vector. Although this system may be convenient for laboratory-based experiments, it adds an extra layer of complexity and difficulty in standardisation should these lines ever be produced on a commercial basis.

4.3.6 ENVELOPE PSEUDOTYPING

There have been many studies involving heterologous envelope pseudotyping of different viral cores. HIV envelope was shown to pseudotype murine vectors although with a significantly lower titre than the native murine amphotropic envelope. More recently, non-retroviral envelopes have been used, particularly the envelope (G protein) of vesicular stomatitis virus (VSV) [58,59]. These appear to pseudotype HIV cores readily and produce a particle that is stable on freezing [58] and that gives a high titre in a CD34

positive bone marrow stem cell population. The combination of a lentiviral core and a VSV envelope has also been shown to deliver genes efficiently to cells in G_0 when tested on growth-arrested cultures. In parallel experiments the titre of murine vectors dropped significantly to near zero. More impressive was the demonstration that highly concentrated (concentration unspecified) VSV-G pseudotyped HIV viral particles injected directly into the mammalian brain were able to effect stable gene transfer into both neurons and glial cells [59]. VSV pseudotyping appears to allow vector particle entry via an endosomal route, which may account for some of the increased efficacy [60]. This is now an area of great interest and gene delivery to a number of tissues *in vivo* has been demonstrated. It is clear, however, that although *in vitro* titres up to 10^9 cfu/ml are claimed there is still a relatively small number of cells transduced *in vivo*. There is also some controversy as to the efficacy of such vectors to deliver genes to important targets such as haemopoietic stem cells [61].

4.3.7 PROTEIN DELIVERY

HIV is one of the first viruses in which there has been a dissection of protein–protein interactions that lead to incorporation of accessory proteins into the virus particle. The protein Vpr, for example, is included in the virus particle due to a specific interaction with the P6 portion of Gag [24] and mutagenesis has identified amino acids involved in this interaction. Similarly, the cell-derived protein cyclophilin interacts directly with CA. Identification of these phenomena has been used to introduce P6 binding regions into heterologous proteins and to demonstrate incorporation of such tagged molecules into virus particles. Although gene therapy and vector systems are usually aimed at delivering nucleic acid sequences, there are clearly many applications for delivery of specific proteins to a cell.

4.3.8 VIRAL TITRES

When full-length viral RNAs incorporate into virus particles even with heterologous genes incorporated either in place of *nef* or substituting for part of the envelope sequence, the efficiency of encapsidation and delivery seems extremely high. This degree of efficiency has never been achieved with any of the known vector systems to date involving vectors that are less than genome length. Even with incorporation of all known packaging signal sequences into a vector including the TAR stem loop, the 5' leader sequence, the *gag* gene, the 3' end of envelope or the RRE alone rarely achieves titres of greater than 10^3 cfu/ml. The reason for this is not clear and it differentiates lentiviral vectors from murine systems, in which encapsidation of vector is achieved at levels equivalent to the wild-type viral RNA. This may reflect a

preference for lentiviruses to encapsidate RNA coding for Gag proteins cotranslationally.

4.4 RANGE OF APPLICATIONS

The potential uses of lentiviral-based vectors were alluded to earlier in the chapter. Obvious roles include the delivery of genes to CD4 positive cells for the gene therapy of HIV infection. This would most likely be directed first at those patients who are already HIV positive in an attempt to provide genetic protection to those CD4 cells in the individuals that were not yet infected. Other diseases affecting lymphocytes and cells of the monocyte/macrophage line might also be targeted.

The use of lentiviruses or lentiviral chimeras to enter non-dividing cells has been demonstrated and there is likely to be an expansion in this area of interest over the next few years. Most individuals involved in vector research have a conception that the perfect vector will be a mixture of components from different viruses, each contributing its own particular property, and that these may ultimately be incorporated into a completely non-viral particle.

4.5 SAFETY

In the United Kingdom, HIV and SIV are category III pathogens and all work involving live viruses or complementary constructs that could regenerate live virus has to be carried out under this level of protective isolation. Work with other lentiviruses (MVV, EIAV) is restricted only by the potential of these pathogens to come into contact with uninfected animals.

As yet, there have been no clinical trial protocols approved using lentiviruses in whole or in part in human gene transfer protocols. There would seem to be little difference in the level of safety required for a vector composed entirely of murine retroviral components compared to one which was chimeric in which part of the chimera involved a lentivirus.

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5 Adenoviral Vectors

SHEILA CONNELLY

5.1 INTRODUCTION

Adenoviral vectors are among the most promising gene transfer vehicles for direct, *in vivo* gene therapy for the treatment of a diverse array of human diseases, including monogenic inherited disorders such as cystic fibrosis, Duchenne muscular dystrophy, and hemophilias A and B, as well as acquired diseases such as cancer (for review see Trapnell and Gorziglia, 1994; Wilson, 1995). In addition, the use of adenoviral vectors for *ex vivo* therapies also has been evaluated. Adenoviral vectors offer many advantages over other gene delivery systems. Adenoviral vectors can transduce a wide spectrum of cell types and do not require division of the target cell for gene transfer or expression. The adenovirus chromosome remains episomal in the transduced cell, thus avoiding the possibility of insertional mutagenesis (Gingsberg, 1984; Horowitz, 1990). Adenoviruses can be rendered replication deficient by deletion of critical viral regulatory genes (Berkner, 1988; Gorziglia *et al.*, 1996), which allow the vectors to accommodate heterologous DNA inserts of more than 8 kb, depending on the extent of the viral gene deletions. Replication-deficient vectors can be produced, in a variety of complementing cell lines, easily and at high titers ($\sim 10^{11}$ infectious units per milliliter). Finally, adenovirus infection has never been associated with any type of tumor in humans, as adenoviruses have been evaluated extensively as live vaccines in millions of individuals (Straus, 1984). The main disadvantage of adenoviral vectors is that the host immune response, in general, appears to limit the duration of transgene expression and the ability to re-administer the vector. Efforts currently are directed toward reducing the immunogenicity of the vectors and to developing strategies to circumvent the host immune response. Within the past several years, numerous reports of successful adenoviral vector-mediated delivery and expression of a wide variety of heterologous genes in several mammalian species have appeared (Trapnell and Gorziglia, 1994; Wilson, 1995). To date, adenoviral vectors are employed in or have been proposed for use in human clinical trials for the treatment of cystic

fibrosis, ornithine transcarbamoylase deficiency, and a diverse array of cancers, including breast, colon, glioblastoma, head and neck, liver, melanoma, neuroblastoma, ovarian, prostate (Marcel and Grausz, 1996), and lung (Cohen-Haguenauer, 1996). However, adenoviral vector-mediated gene therapy is at an early stage and nearly all of the studies consist of phase I trials, with the goal of establishing safety, rather than efficacy, of the procedures.

5.2 STRUCTURE AND GENOMIC ORGANIZATION OF HUMAN ADENOVIRUSES

Human adenoviruses are non-enveloped DNA viruses belonging to the parvoviridae family (reviewed by Ginsburg, 1984; Horowitz, 1990). The virion is 80–90 nm in diameter with a spiked, icosahedral morphology and a molecular mass of $175\text{--}185 \times 10^6$ Da. Adenoviruses have been classified serologically into nearly 50 distinct serotypes, subgrouped A through G (Wadell, 1984). While viruses of the subgroups A and B have been shown to have oncogenic potential in newborn rodents, viruses used as gene transfer vectors belong to the non-tumorigenic subgroup C. Subgroup C adenoviruses cause mild respiratory disease in humans, and account for 5–15% of occurrences of the 'common cold' (Straus, 1984).

Since the isolation of adenoviruses over 40 years ago (Rowe *et al.*, 1953), knowledge of the adenovirus genetic system has increased dramatically, in part due to early interest in adenovirus as a model of eukaryotic gene expression (Ginsburg, 1984; Horowitz, 1990). Adenovirus infects target cells by attachment to the coxsackie and adenovirus receptor (CAR) on the cell surface (Bergelson *et al.*, 1997), internalization via clathrin-coated pits into endosomes, escape of the virion into the cytoplasm by endosomolysis, translocation to the nuclear membrane via nuclear targeting signals within the capsid polypeptides, and transport of the viral genome into the cell nucleus, where the viral genome remains episomal.

The genome consists of a 36 kb linear, double-stranded DNA molecule, which contains, covalently attached at each 5' end, a 55 kDa terminal protein important for viral DNA replication. The viral DNA contains short, inverted terminal repeats (ITRs) at each end of the genome that are required for DNA replication. The viral genome is organized into four distinct early regions, termed E1–E4, and five alternatively spliced late regions, L1–L5, based on expression before or after the initiation of viral DNA synthesis, in addition to several minor intermediate and/or late transcription units (Figure 5.1). In general, early gene products alter host cell biology to support virus production, while the late genes encode most of the structural proteins which comprise the virion and aid in viral assembly. Immediately upon entry of the viral genome into the nucleus, the E1 region is actively transcribed by cellular

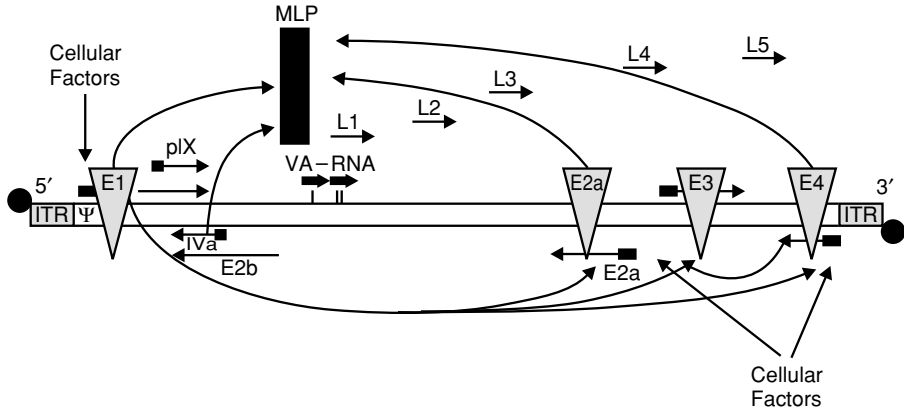


Figure 5.1. Schematic representation of the adenovirus genome and regulation of transcription. The adenoviral genome is represented by the double parallel lines, and transcription units are represented by the horizontal arrows. The arrowheads indicate the direction of transcription. Curved arrows represent transactivation pathways. Intermediate and/or minor transcription units, pIX, IVa, and VA-RNA (I-III), are displayed. The viral inverted terminal repeat regions are indicated (ITR) as well as the 55 kDa terminal proteins covalently attached to the 5' ends of the genome. Both ITR regions and the presence of terminal protein are required for efficient viral replication. The majority of first-generation adenoviral (Av1) vectors contain deletions in both the E1 and E3 regions; however, a few Av1 vectors contain only the E1 region deletion. Removal of the E1 region renders the virus replication impaired, as the E1 gene products upregulate the majority of adenoviral transcription units, such as E2, E3, E4, and the major late promoter (MLP), indicated by the curved arrows. E1, E2, and E4 are activated by host cell endogenous transcription factors. Third-generation vectors (Av3) contain, in addition to deletions of E1 and E3, a partial deletion of the E2a coding region. Vectors with combined deletions of E1/E3/E4 have been generated also. These further attenuated vectors reduce expression of the late transcription units by preventing upregulation of the MLP by the E2a or E4 gene products, respectively. Large triangles represent deletions of adenoviral regions; (■), promoters; (●), terminal protein.

transcription factors, and codes for proteins directly involved in the activation of the remaining early regions, E2, E3, and E4. The E1 gene products, encoded in the E1a and E1b regions, have been implicated in virus-induced transformation of cultured cells (Van der Eb *et al.*, 1977). The E2 region encodes proteins required for viral DNA replication, including a single-stranded DNA binding protein (E2a) and both the viral DNA polymerase and the 55 kDa terminal protein (E2b). The E3 region is composed of a series of transcription units involved in evading host defense mechanisms that act to eliminate virus infected cells, and is dispensable for virus replication (Wold and Gooding, 1991). E4 gene products are involved in the regulation of viral and cellular protein expression, viral DNA replication, viral late

mRNA accumulation and protein synthesis, and the corresponding down-regulation of host protein synthesis. A recent report has demonstrated that an E4 protein, encoded in open reading frame 6, has oncogenic potential, similar to that described for the E1b gene product (Moore *et al.*, 1996). Expression of the early genes leads to DNA replication, approximately eight hours after infection, and subsequent activation of the late genes under the transcriptional control of the major late promoter, production of virus progeny, and finally, death of the host cell and virus release.

5.3 DESIGN AND CONSTRUCTION OF REPLICATION-DEFECTIVE HUMAN ADENOVIRAL VECTORS

Replication-deficient adenoviral vectors, similar to other viral vectors, are composed of the virion structure surrounding a modified viral genome. To date, most vector particles are based on the wild-type capsid structure which, in addition to protecting the viral DNA, provides the means to bind and enter (transduce) target cells. However, the viral genome has been modified substantially. These changes are designed to disable growth of the virus in target cells, by deleting viral functions critical to the regulation of DNA replication and viral gene expression, while maintaining the ability to grow in available packaging or helper cells. Deletion of such sequences provides space within the viral genome for insertion of exogenous DNA that encodes and enables appropriate expression of the gene of interest (transgene).

The subgroup C adenoviruses, serotypes 2 and 5 (Ad2 and Ad5), are among the best studied adenoviruses, and the viruses used most commonly as gene transfer vectors. The vast majority of adenoviral vectors for gene therapy are E1 replacement vectors, where the transgene is inserted in place of the E1 region. This E1 region deletion includes the entire E1a gene and approximately 60% of the E1b gene. The vectors retain the immediate 5' end of the viral genome, including the left inverted terminal repeat (ITR) and encapsidation signal (ψ), sequences required for packaging, and the overlapping E1 enhancer region, in addition to the remainder of the viral genome (Figure 5.1). As the E1 gene products lead to sequential activation of the major transcription units, deletion of this region greatly reduces early and late gene expression and renders the virus severely replication impaired (Berkner, 1988). To provide more space within the adenoviral vectors for insertion of the transgene, the E3 region, not required for viral replication or growth, is also frequently deleted. Occasionally, the transgene is inserted into this E3 region deletion. Adenoviral vectors lacking only E1 and E3 regions are referred to as first generation, or Av1, vectors. Adenoviruses can effectively package DNA up to 105% of the genome size (Bett *et al.*, 1993), allowing the accommodation of up to 8 kb of exogenous DNA in E1/E3 deleted Av1 vectors.

Av1 vectors have been constructed in several ways. The most commonly employed technique is through homologous recombination of the viral genome with a plasmid bearing the transgene (Berkner, 1988). Briefly, the transgene transcription unit is inserted into a plasmid containing a segment of the viral genome. This plasmid is co-transfected into a permissive cell line (see below), with appropriately prepared viral DNA, by conventional DNA transfer techniques. In the cells, homologous recombination results in the rescue of the cloned viral sequences and the transgene into the viral genome, thus generating the *recombinant* adenoviral vector (Figure 5.2). To generate Av1 vectors with the transgene insertion in the E1 region, the plasmid must contain the left end of the viral genome, including the ITR and encapsidation signal, the transgene expression cassette, and approximately 1 kb or more (Berkner and Sharp, 1983) of downstream viral DNA sequence. The viral DNA used in the co-transfection is prepared by restriction enzyme cleavage to remove the left end of the viral genome. Preparation of the viral DNA in this manner reduces the infectivity of the parental viral DNA and therefore enhances the efficiency of isolation of recombinant vectors resulting from *in vitro* recombination. The large DNA fragment (~34 kb) isolated from the Ad5 E3 deletion mutant, dl327 (Thimmappaya *et al.*, 1982), cleaved with the restriction enzyme Cla I and gel-purified, is used routinely for this purpose (Smith *et al.*, 1993). In a similar manner, heterologous DNA can be inserted into the E3 region. In this case, however, the plasmid contains the right end portion of the adenovirus genome, and the viral DNA is prepared by restriction enzyme digestion to remove the right end of the genome. Alternatively, restriction enzyme digested viral DNA isolated to retain the 55 kDa terminal protein covalently attached to the 5' end of the viral genome has been used in co-transfection procedures (Sharp *et al.*, 1976). While the infectivity of viral DNA containing terminal protein is at least 10-fold higher than protease-digested genomic DNA (Sharp *et al.*, 1976), a high background of non-recombinant virus due to the presence of small amounts of highly infectious uncleaved viral DNA may be detected (Berkner and Sharp, 1983). Following transfection, the resulting viral plaques are isolated, expanded, and screened by restriction analysis, Southern blotting, and/or transgene expression to identify the desired recombinant vector (for detailed protocols, see Graham and Prevec, 1991).

Other methods employed to generate adenoviral vectors include the direct cloning of plasmid sequences into the adenoviral genome via appropriate restriction sites, with subsequent transfection of permissive cells with the *in vitro* ligated DNA. Such a procedure was one of the original methods described for the generation of adenovirus host-range mutants (Stow, 1981). However, due to the large size of the adenovirus genome, the availability of unique or infrequent restriction sites is limited.

A significant problem associated with the generation of adenoviral vectors

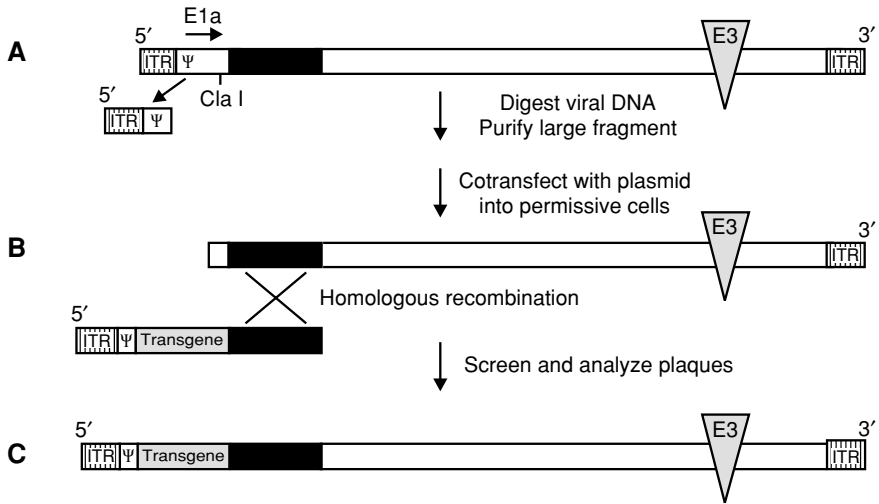


Figure 5.2. Generation of recombinant adenoviruses. (A) Adenoviral genomic DNA is prepared by Cla I digestion of the E3 region deletion mutant Ad5-dl327 to remove the left end of the viral genome, and to render the viral DNA non-infectious. (B) A plasmid containing a viral ITR and packaging signal, the transgene transcription unit, and one kilobase or more of adenoviral DNA is co-transfected with the prepared viral DNA into permissive cells. (C) As a result of intercellular homologous recombination, a recombinant E1/E3 deleted adenoviral vector containing the transgene replacing the viral E1 genes is generated. The horizontal arrow represents the E1 transcription unit. (▤▤▤), viral ITR; (Ψ), viral packaging signal; (▒), exogenous transgene transcription unit; (■), homologous recombination region; (□), adenoviral genomic DNA. Large triangles represent deletion of the adenoviral E3 region.

by direct ligation or homologous recombination of plasmid and viral DNA is the rescue of parental virus caused by the presence of contaminating infectious parental viral DNA. The recovery of the recombinant virus, in the presence of such background, can be difficult, especially if the engineered vector grows more slowly than the competing, parental virus. Several clever approaches have been utilized to solve this problem. For example, parental vectors with impaired replication have been employed to eliminate the possibility of a growth advantage over the desired recombinant (Berkner, 1988). Likewise, an Av1 vector expressing a conditional lethal phenotype was developed by the inclusion of the herpes simplex virus thymidine kinase (TK) gene into the viral genome (Imler *et al.*, 1995). The expression of the TK gene product prevented viral replication in the presence of the nucleoside analog ganciclovir, allowing for the selection of only the recombinant vectors in which the TK gene had been replaced (Imler *et al.*, 1995). Another approach is to circumvent the need for infectious viral DNA with

the use of plasmids that comprise the entire adenovirus genome (Ghosh-Choudhury *et al.*, 1986; Bett *et al.*, 1994). Infectious virus can be generated upon transfection of one or more adenoviral DNA-containing plasmids. However, the transfection efficiency of plasmid DNA is much lower than that of viral DNA (Ghosh-Choudhury *et al.*, 1986; Bett *et al.*, 1994). Similarly, the complete genome of Ad2 was constructed as a yeast artificial chromosome (YAC), and shown to be infectious (Ketner *et al.*, 1994). Using conventional yeast genetic techniques, the viral sequences contained in the YAC can be modified, and adenoviral vectors recovered from the YAC clones (Ketner *et al.*, 1994).

The transgene transcriptional unit consists of the elements required to enable appropriate expression of the transgene such as the promoter, the gene of interest, and a polyadenylation signal, and, in most instances, is designed to maximize the expression of the exogenous gene. A large variety of promoters have been utilized for transgene expression, the choice of which depends on the application and the target tissue. Strong, constitutively expressed viral promoters such as the adenovirus major later promoter (Stratford-Perricaudet *et al.*, 1990), the Rous sarcoma virus promoter (Stratford-Perricaudet *et al.*, 1992; Smith *et al.*, 1993), the cytomegalovirus (CMV) promoter (Herz and Gerard, 1993) and a hybrid CMV enhancer/ β -actin promoter (Kozarsky *et al.*, 1993) have been incorporated into recombinant adenoviral vectors. More recently, the use of cellular, tissue-specific promoters such as the liver-specific albumin promoter (Connelly *et al.*, 1995, 1996a,b,c), lung-specific cystic fibrosis transmembrane conductance regulator promoter (Imler *et al.*, 1996; Suzuki *et al.*, 1996), the cardiac muscle-specific myosin light chain-2 promoter (Rothmann *et al.*, 1996), and the hepatoma-specific α -fetoprotein promoter (Kaneko *et al.*, 1995; Arbuthnot *et al.*, 1996) has been described. Finally, regulatable promoters responsive to hormonal (Hayashi *et al.*, 1994) or pharmacological agents (Suzuki *et al.*, 1996) have been incorporated into adenoviral vectors. The inclusion of tissue-specific and/or regulatable promoters to the transgene expression cassette avoids the unknown consequences of overexpression of genes in tissues other than the targeted organ, and may, therefore, increase the safety of such vectors. An additional approach shown to increase the potency of the transgene in adenoviral vectors is the introduction of genomic elements into the expression cassette. For example, the addition of an intron to the human factor VIII (FVIII) cDNA boosted *in vivo* expression approximately 10-fold (Connelly *et al.*, 1996b), and the inclusion of the human factor IX (FIX) truncated first intron and 5' and 3' untranslated regions to the human FIX cDNA functioned synergistically to increase human FIX plasma levels in transduced mice approximately 2000-fold (M. Kaleko, unpublished). Finally, a variety of signals have been used to direct polyadenylation such as the simian virus 40 polyadenylation signal.

5.4 PROPAGATION AND PURIFICATION OF ADENOVIRAL VECTORS

The propagation of Av1 adenoviral vectors, rendered almost completely replication defective by the deletion of the E1 region, requires the generation of cell lines to complement the E1 functions *in trans*. Several human cell lines that constitutively express the E1 proteins have been established. To date, the most widely used cell line, 293, consists of human embryonic kidney cells transformed with sheared Ad5 DNA that express the left 11% of the Ad5 genome (Graham *et al.*, 1977). While 293 cells allow replication of Av1 vectors to high titers, this cell line is not ideal for large-scale vector production. Recombination between homologous E1 region sequences encoded in the vectors with those inserted in the 293 cell genome has the potential to generate replication-competent adenoviruses (RCA) (Lochmüller *et al.*, 1994). RCA are to be avoided as uncontrolled replication of the 'reverse recombinant' also would allow replication of the vector. Furthermore, the presence of RCA in preparations of adenoviral vectors was shown to induce significant tissue damage *in vivo* (Lochmüller *et al.*, 1994). The generation of RCA may be prevented by elimination of sequence homology between the vector DNA and the adenovirus sequences in the genome of the complementing cells. Recently, the development of Av1 vectors containing more extensive deletions of the E1 region, and alternative cell lines for the propagation of these vectors have been described (Fallaux *et al.*, 1996; Imler *et al.*, 1996). Cell lines that express, in addition to the adenovirus E1 region, E2a or an E4 protein encoded in open reading frame 6 (orf6) of the E4 region have been generated and used to propagate adenoviral vectors containing deletions of the E2a or the E4 regions (see below) (Armentano *et al.*, 1995; Wang *et al.*, 1995; Gao *et al.*, 1996; Gorziglia *et al.*, 1996; Yeh *et al.*, 1996). As constitutive expression of adenoviral proteins, in many instances, is toxic, the development of cell lines that express multiple adenoviral proteins is difficult (Wang and Finer, 1996). Unlike retroviral vectors, the stability of the adenovirus virion allows extensive purification and concentration without significant loss of activity. Procedures for adenoviral vector purification involve harvest and disruption of infected cells using multiple freeze and thaw cycles (Smith, 1995), or sonication (Kanegae *et al.*, 1994), and removal of the cell debris by centrifugation. Vector is purified and concentrated in one to three CsCl centrifugation steps, followed by dialysis or chromatography. However, for large-scale manufacturing, chromatographic methods which avoid CsCl centrifugation are desirable. Vector concentration is determined spectrophotometrically, to evaluate particle number, and biologically, to measure infectivity by gene transfer or plaque assay (Mittereder *et al.*, 1996). For use in human clinical trials, preparations must meet a Food and Drug Administration (FDA) requirement of a particle to infectious unit ratio of less than 100

(Smith, 1995; Mittereder *et al.*, 1996). Concentrated vector preparations containing 10^{11} plaque forming units per milliliter can be obtained routinely, several orders of magnitude greater than possible with retroviral or adeno-associated viral vectors. Vector preparations are tested extensively for RCA by E1-specific polymerase chain reaction (PCR) analysis (Tolstoshev *et al.*, 1994) and plaque assay. The current FDA requirement for clinical lots of adenovirus is one or fewer RCA per vector dose (Smith, 1995).

5.5 *IN VIVO* ADENOVIRUS-MEDIATED GENE TRANSFER

Within the past several years, extensive evaluation of the efficacy and safety of Av1 vectors administered *in vivo* to a wide spectrum of animal species and humans has resulted in increased understanding of adenoviral vector biology (Trapnell and Gorziglia, 1994; Wilson, 1995). Adenoviral vector-mediated expression of a diverse array of transgenes, including reporter genes, such as β -galactosidase and luciferase, has been detected in numerous tissues, the most highly studied of which are the lung and the liver. Adenovirus-mediated gene expression is dependent upon the animal model, the vector, and dose utilized, but in general initial expression is high. The duration of gene expression, however, varies widely. In immunocompromised or immunologically immature mice, expression is generally longer term than that detected in adult mice, where expression declines greatly within two to three weeks, suggesting that the immune system limits vector persistence (Trapnell and Gorziglia, 1994; Wilson, 1995). However, after intravenous delivery of Av1 vectors to normal adult mice, long-term expression of human blood clotting FVIII (Connelly *et al.*, 1996a) and FIX (M. Kaleko, unpublished) has been reported. Transduction of muscle with Av1 vectors also, in some cases, resulted in persistent expression (Stratford-Perricaudet *et al.*, 1992; Ragot *et al.*, 1993; Vincent *et al.*, 1993; Tripathy *et al.*, 1996).

Interest in developing gene therapy for the two most common hereditary lung diseases, α_1 -antitrypsin (α_1 -AT) deficiency and cystic fibrosis (CF), has resulted in extensive analysis of adenoviral-mediated delivery of reporter genes, α_1 -AT, and the human cystic fibrosis transmembrane conductance regulator (CFTR) cDNA to the lung. The initial studies were performed using the cotton rat lung as the animal model, as the cotton rat has been shown to display a sensitivity to infection with adenovirus similar to that seen in humans. Tracheal instillation of an α_1 -AT-encoding Av1 vector resulted in expression of α_1 -AT (Rosenfeld *et al.*, 1991). Similarly, *in vivo* administration of a CFTR-encoding adenoviral vector into the airway of the cotton rat resulted in CFTR expression and CFTR RNA detectable for six weeks (Rosenfeld *et al.*, 1992). Despite the high efficiency of transgene delivery to the lung, the duration of gene expression was short term, and associated with an acute

cellular inflammation within the pulmonary parenchyma in the cotton rat (Yei *et al.*, 1994b). Studies in non-human primates (Simon *et al.*, 1993) have demonstrated similar host immune responses, but with a more protracted time course. Administration of the CFTR-encoding Av1 vector to the nasal epithelia of human CF patients resulted in short-term correction of the chloride secretory defect with no evidence of vector-associated toxicity (Zabner *et al.*, 1993). In a separate study, an Av1 CFTR vector was administered to the nose and lung of CF patients; expression of CFTR RNA was detected in the nose two days after treatment, and one patient who received a higher vector dose to the lung developed an acute self-limited febrile response associated with a rise in adenovirus antibody titer and lung interleukin-6 levels (Crystal *et al.*, 1994).

A second major target organ for *in vivo* gene therapy is the liver. Many genes, including those encoding blood clotting factors, metabolic enzymes, and lipoproteins, are candidates for liver gene therapy. Notably, peripheral vein administration of adenoviral vectors to mice (Smith *et al.*, 1993), dogs (Connelly *et al.*, 1996c), and non-human primates (T.A.G. Smith, unpublished) results in efficient transduction of hepatocytes, demonstrating the feasibility of non-invasive, systemic adenoviral vector delivery for the treatment of liver disorders. Much progress has been made recently in adenoviral vector-mediated gene therapy for the hemophilias A and B, deficiencies of blood coagulation FVIII and FIX. Intravenous administration of low doses of potent Av1 vectors encoding the human blood clotting FVIII and FIX to normal adult mice resulted in expression of therapeutic levels of the clotting factors for at least five months (Connelly *et al.*, 1996a), and over one year (M. Kaleko, unpublished), respectively. However, when high, hepatotoxic, doses of the vectors were administered, the initial high levels of clotting factor expression declined rapidly to background suggesting that dose-dependent vector toxicity limited vector persistence (Smith *et al.*, 1993; Connelly *et al.*, 1995, 1996a). Administration of the FVIII adenoviral vector to FVIII-deficient dogs resulted in complete correction of the hemophiliac phenotype and high-level expression of human FVIII (Connelly, 1996c). However, FVIII expression in the dogs was short term due to the development of human FVIII inhibitory antibodies (Connelly *et al.*, 1996c). Treatment of hemophilia B dogs with a canine FIX-encoding Av1 vector resulted in transient phenotypic correction of the bleeding disorder, although, in this case, the presence of canine FIX-specific antibodies was not detected (Kay *et al.*, 1994).

In other models of liver-targeted disease, Av1 vectors including those encoding an ornithine transcarbamylase gene (Stratford-Perricaudet, 1990; Morsy *et al.*, 1993), a phenylalanine hydroxylase gene (Fang *et al.*, 1994), an apolipoprotein E gene (Stevenson *et al.*, 1995), and the human low density lipoprotein (LDL) receptor gene (Ishibashi *et al.*, 1993) have been used to treat genetically engineered mice containing knockouts of the studied gene.

In all cases, phenotypic correction of the defect after adenoviral vector treatment was reported. However, in most instances, the therapeutic effect was transient. Similarly, treatment of the hyperlipidemic Watanabe rabbit with the LDL receptor-encoding Av1 vector resulted in significant lowering of the cholesterol levels lasting for three weeks (Kozarsky *et al.*, 1994).

Av1 vectors have been evaluated and shown to efficiently transduce a wide variety of tissues and organs in addition to the lung and liver, such as heart (Kass-Eisler *et al.*, 1993), skeletal and cardiac muscle (Stratford-Pericaudet *et al.*, 1992; Kass-Eisler *et al.*, 1993; Ragot *et al.*, 1993; Vincent *et al.*, 1993), bone marrow (Mitani *et al.*, 1994), brain (Le Gal La Salle *et al.*, 1993), CNS (Bajocchi *et al.*, 1993; Davidson *et al.*, 1993), endothelial cells (Lee *et al.*, 1993; Lemarchand *et al.*, 1993), kidney (Moullier *et al.*, 1994), retinal cells (Jomary *et al.*, 1994), and solid tumors (Haddada *et al.*, 1993; Brody *et al.*, 1994; Wills *et al.*, 1994).

Adenoviral vector-mediated therapy for acquired disorders, such as cancer, have also been explored using two different strategies, inducement of tumor cell-specific cytotoxicity and enhancement of existing host antitumor immunity. Tumor cell cytotoxicity has been induced by treatment of tumor tissue with adenoviral vectors encoding p53 (Wills *et al.*, 1994; Zhang *et al.*, 1995), or utilization of a combination of a herpes thymidine kinase gene and ganciclovir to successfully treat pre-established tumors in murine models (Chen *et al.*, 1995; Yee *et al.*, 1996). Tumor vaccination approaches include the use of an Av1 vector encoding an interleukin-2 cDNA, which was shown to enhance antitumor immunity in mice (Haddada *et al.*, 1993; Addison *et al.*, 1995; Cordier *et al.*, 1995; Huang *et al.*, 1996).

5.6 CIRCUMVENTING THE HOST IMMUNE RESPONSE TO *IN VIVO* ADENOVIRAL GENE TRANSFER

Despite the high transduction and transgene expression efficiency obtained with first-generation adenoviral vectors, the duration of gene expression, in many cases, is transient. This loss of expression is associated with direct toxicity of the vector (Connelly *et al.*, 1996a) and infiltration of inflammatory and immune cells (Yei *et al.*, 1994a; Yang *et al.*, 1994a,b, 1995a), resulting in destruction of the transduced cells. Characterization of cells transduced with Av1 vectors has revealed that although expression of most adenoviral genes is severely attenuated, a low level of expression of viral proteins (viral backbone gene products) can be detected (Mittereder *et al.*, 1994; Yang *et al.*, 1994a,b). These observations led to the suggestion that a cytotoxic T lymphocyte (CTL) response directed against cells expressing viral backbone genes resulted in elimination of the genetically modified cells (Yang *et al.*, 1994ab, 1995a, 1996c). Therefore, further attenuation of viral gene expression

may reduce host immune responses to transduced cells and increase the duration of transgene expression. Indeed, the development of a second-generation, Av2, adenoviral vector with a temperature-sensitive 72-kDa DNA-binding protein, encoded in the E2a region of the virus backbone, allowed prolonged expression in the livers of immune competent adult mice and was associated with a reduced CTL response (Engelhardt *et al.*, 1994ab, Yang *et al.*, 1994c; Ye *et al.*, 1996). However, analysis of the Av2 vector in a different mouse strain and in hemophilia B dogs revealed no difference in transgene expression persistence compared to similar Av1 vectors (Fang *et al.*, 1996). Recently, a third-generation, Av3, vector, containing deletions in the E1, E2a, and E3 regions (Figure 5.1), has been generated and characterized *in vitro* and shown to be improved over Av1 and Av2 vectors with respect to the potential for vector DNA replication and viral late protein expression (Gorziglia *et al.*, 1996). In addition, adenoviral vectors containing combined deletions in the E1 and E4 regions have been described (Armentano *et al.*, 1995; Wang *et al.*, 1995; Gao *et al.*, 1996; Yeh *et al.*, 1996). Subsequent generation adenoviral vectors containing more extensive backbone gene deletions are, no doubt, in progress, and require, in addition, the development of complementing cell lines to supply the missing gene products *in trans*. Ultimately, the use of adenoviral vectors containing only the essential adenovirus packaging signals and the transgene expression cassette may become feasible, and such vectors have been reported (Clemens *et al.*, 1996; Haecker *et al.*, 1996; Kochanek *et al.*, 1996; Lieber *et al.*, 1996). However, these vectors are grown in the presence of a helper virus which is difficult to separate completely from the vector. A packaging cell line that possesses all the viral complementing functions, although difficult to generate, would allow production of the vector without helper contamination. These 'gutless' adenoviral vectors have the potential for minimizing cytotoxicity and cellular immune responses as well as allowing the accommodation of larger (up to 35 kb) exogenous DNA fragments.

Alternative methods for reducing the cellular immune response to adenoviral transduced cells resulting in sustained transgene expression include the use of immunosuppressive reagents such as cyclosporin A (Fang *et al.*, 1995), FK506 (Lochmüller *et al.*, 1995, 1996; Vilquin *et al.*, 1995), and cyclophosphamide (Jooss *et al.*, 1996). However, the prolonged use of immunosuppressants may induce severe side effects. As an alternative to immunosuppressant drugs, monoclonal antibodies, directed against proteins involved in T cell activation, also were effective in reducing cell-mediated immunity (Kay *et al.*, 1995; Guérette *et al.*, 1996; Yang *et al.*, 1996b). Therefore, the use of improved adenoviral vectors containing more extended deletions of viral backbone genes in combination with immunosuppressive regimens designed to block cell-mediated immunity may allow more persistent transgene expression.

Another host immune-mediated limitation to the use of adenoviral vectors is that repeated vector administration has been unsuccessful, as a result of a humoral immune response to the viral capsid proteins (Smith *et al.*, 1993; Kay *et al.*, 1994; Kozarsky *et al.*, 1994; Yei *et al.*, 1994a). The generally transient nature of adenoviral vector-mediated transgene expression will require multiple administrations of the vector, especially for the treatment of chronic illnesses such as cystic fibrosis and hemophilia, where life-long therapy will be required. Studies by Smith *et al.* (1996) have demonstrated that the immune response to a systemically administered adenoviral vector is dose dependent and can be modulated by transient immunosuppression with cyclophosphamide or deoxyspergualin (DSG) at the time of initial vector treatment to allow effective repeated treatment. More recently, using low-dose combination immunotherapy, at least three efficacious adenoviral vector treatments were achieved (T.A.G. Smith, unpublished). In addition, repeated vector delivery has been achieved via immunosuppression strategies designed to block T and B cell activation (Yang *et al.*, 1995b, 1996a), and by the induction of tolerance by vector administration to neonatal mice (Walter *et al.*, 1996). Finally, the use of adenoviral vectors derived from different virus serotypes has been proposed, as adenovirus neutralizing antibodies developed with the administration of one virus serotype do not cross-react or prevent transduction with adenovirus of a second serotype (Kass-Eisler *et al.*, 1996; Mastrangeli *et al.*, 1996). However, such a strategy would involve the generation and characterization of multiple vectors encoding the gene of interest.

5.7 SUMMARY

Adenoviral vectors currently represent the most efficient means to transfer an exogenous gene to a large spectrum of target cells *in vivo*. Numerous demonstrations of efficacious adenoviral vector-mediated delivery of a diverse array of transgenes, in several animal species and humans have been reported. In general, initial transgene expression is extremely efficient, but transient, in many cases lasting less than one month. Much effort has been directed at overcoming the obstacles that may restrict use of this vector system to treat human disease effectively. The major hurdles are (i) the cellular immune response to transduced cells that express low levels of viral backbone genes resulting in cell elimination; (ii) humoral immunity to the viral capsid that limits repeated vector administration; and (iii) the potential for generation of contaminating RCA in the production of vectors for clinical use. While much work still needs to be done, many advances have been made toward overcoming each of these obstacles. Subsequent generation vectors containing more extended deletions (Av2, Av3, gutless) and cell lines to

propagate these vectors have been generated which may reduce host immune responses to transduced cells and therefore increase the duration of transgene expression. A variety of novel strategies have been developed to subdue the immune system, which, taken together with improved vector design, may allow persistent and efficacious repeated vector administration. Finally, the generation of new cell lines and improved vector purification and wild-type adenovirus detection techniques have greatly reduced the possibility of the generation of contaminating RCA. To date, human clinical trials utilizing the early generation, Av1, adenoviral vectors, have revealed that human gene transfer is feasible. The Av1 vectors may find successful application in the treatment of human disease where short-term expression and single dosing is adequate, such as cancer vaccine therapies. Alternatively, future clinical trials using the improved gene transfer strategies and more attenuated vectors may demonstrate efficacious, long-term gene therapy for treatment of a diverse range of human diseases.

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6 Adeno-Associated Viral Vectors

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6.1 INTRODUCTION

Adeno-associated virus (AAV) is a non-pathogenic human DNA virus with a unique profile of biological properties that have been of interest to molecular virologists for many years (Berns, 1990; Carter, 1990; Carter *et al.*, 1990). Recently, AAV has also attracted interest as a vector for gene transfer (Carter, 1992; Flotte, 1993a; Hermonat and Muzyczka, 1984; Tratschin *et al.*, 1984). In a general sense, AAV is unique among viruses currently being used for gene transfer in that it is a native human virus which is not known to cause disease, and may, in fact, suppress the induction of tumors by other viruses (Cukor *et al.*, 1975; DeLaMaza and Carter, 1981; Hermonat, 1989, 1991; Khelif *et al.*, 1991; Kirschstein *et al.*, 1968; Labow *et al.*, 1987; Mayor *et al.*, 1973; Ostrove *et al.*, 1981). There is a natural enthusiasm to develop therapeutic applications of a virus which is naturally symbiotic with its human host. This must be tempered by careful attention to how the biology of the virus may be altered as its genome is re-engineered to make it into a vector.

6.2 BIOLOGY OF AAV

6.2.1 AAV TAXONOMY AND NATURAL HISTORY

AAV was originally identified as a contaminant of adenovirus cultures. Multiple serotypes of AAV have since been identified, including human AAV serotypes 1, 2, 3, and 5, simian AAV serotype 4, as well as bovine, canine and avian AAV (Blacklow, 1988). AAV is a member of the dependovirus genus of the family Parvoviridae. Like other parvoviruses, AAV exists as a non-enveloped icosahedral particle with a diameter of approximately 20 nm (Figure 6.1).

AAV has never been shown to cause any human disease, despite a high seroprevalence rate. AAV serotypes 2 and 3 have been identified in throat

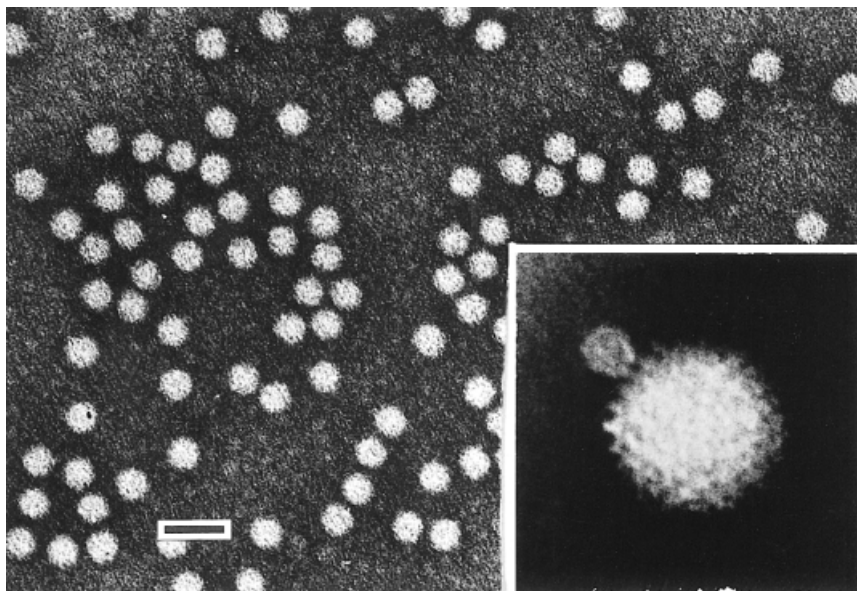


Figure 6.1. Electron micrograph of AAV particles. A field of CsCl gradient-purified ($\rho = 1.41$ g/ml) AAV2 particles is shown, demonstrating the icosahedral shape of the 20 nm virions (magnification = 40 000X). The inset shows a larger adenoviral particle adjacent to an AAV particle from the 1.36 g/ml band (magnification = 120 000X).

and anal swab specimens from otherwise healthy children during a concomitant nursery school outbreak of adenovirus- (Ad-) induced diarrhea (Blacklow *et al.*, 1971). There were no differences observed between the clinical syndromes in individuals infected with AAV and Ad as compared with those infected with Ad alone. Because AAV can also exist in a latent state in human cells, its potential role in neoplastic processes has also been investigated. Surprisingly, AAV seropositivity was inversely correlated with risk for virus-induced cervical carcinoma (Cukor *et al.*, 1975). Studies in animal and tissue culture models of tumorigenesis have confirmed that the AAV-*rep* gene can function as a tumor suppressor (Hermonat, 1991; Kleif *et al.*, 1991; Labow *et al.*, 1987).

6.2.2 THE STRUCTURE OF AAV AND ITS GENOME

The AAV2 genome has been cloned (Laughlin *et al.*, 1983; Samulski *et al.*, 1982), sequenced (Srivastava *et al.*, 1983), and characterized in detail (Figure 6.2). The termini consist of the 145-nucleotide inverted repeat sequences (inverted terminal repeats; ITRs). The outer 125 nucleotides of each ITR form a palindrome which can assume a hairpin configuration in the single-stranded state.

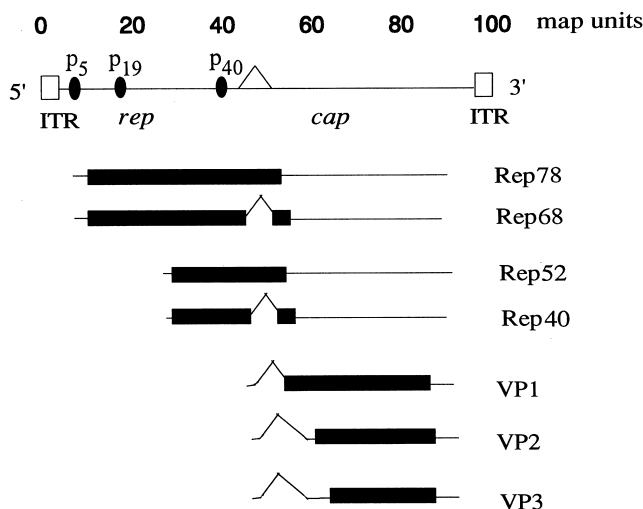


Figure 6.2. Structure of the AAV genome. The AAV2 genome is represented with a 100 map-unit scale (1 map-unit = 1% of genome size, approximately 47 bp). The open boxes represent the inverted terminal repeats (ITRs). The transcription promoters (p₅, p₁₉, p₄₀) are depicted as solid ellipses. The polyadenylation signal is at map position 96. RNA transcripts from AAV promoters are shown below the DNA map with the introns indicated by carets. Protein coding regions are depicted as solid boxes. The three capsid proteins are VP1, VP2, and VP3; the four Rep proteins are Rep78, Rep68, Rep52, and Rep40.

The ITRs contain all *cis*-acting functions required for DNA replication, packaging, integration, and subsequent excision and rescue (Samulski *et al.*, 1989). Also within the ITRs are several transcriptional elements, including Sp1 sites and an initiator (*inr*) site for transcription of RNA (Flotte *et al.*, 1993a). The role of these functions in the natural virus life cycle is unknown. The ITRs also contain binding sites for Rep68 protein (see below), which may be important for the processes of terminal resolution and site-specific integration.

Internal to the ITRs are two viral genes: *rep*, which encodes functions required for replication, and *cap*, which encodes structural proteins of the capsid. The *rep* gene is transcribed from two promoters, the p₅ promoter and the internal p₁₉ promoter. By utilizing each of these promoters with both spliced and unspliced RNA transcripts, a total of four Rep proteins are produced. These have been designated Rep78, Rep68, Rep52, and Rep40 based on their apparent molecular weights. Rep78 and Rep68 have a number of properties, including: (i) DNA binding to a specific Rep-recognition sequence (*rrs*) within each ITR (McCarty *et al.*, 1994a,b), (ii) DNA helicase activity, (iii) site-specific, strand-specific endonuclease activity for AAV-ITRs during viral DNA replication and rescue, (iv) DNA binding to *rrs* sequences

within the preferred chromosome 19 integration sequence (Weitzman *et al.*, 1994), and (v) transcriptional repressor and activator functions (Antoni *et al.*, 1991; Beaton *et al.*, 1989; Kyostio *et al.*, 1994). The first three of these functions appear to be important for normal replication in a productive life cycle. Binding to the *rrs* on chromosome 19 may be important in the latent phase of the life cycle. The transcription regulation functions are important in suppressing viral gene expression during latency and activating it during the replicative phase. Rep78 and Rep68 also modulate transcription from heterologous promoters of other viruses and from cellular genes. This latter activity may also be responsible for the Rep78/68 effect as a suppressor of tumorigenesis (Hermonat, 1991; Khelif *et al.*, 1991). The biochemical functions of Rep52 and Rep40 are less well defined, although these gene products are required for accumulation of single-stranded DNA copies during a productive infection.

The *cap* gene is transcribed from the p_{40} promoter to generate three protein products, VP1, VP2, and VP3, with approximate molecular weights of 85 kDa, 72 kDa, and 61 kDa, respectively. By use of two different splice acceptor sites, two different transcripts are produced: a minor transcript which codes for VP1 and a major transcript which codes for VP2 and VP3 (Trempe and Carter, 1998). These three proteins differ in the length of their amino terminus, but are identical throughout the VP3 coding region. While VP3 accounts for 84% of the capsid protein, all three are required for complete particle assembly.

6.2.3 THE AAV LIFE CYCLE

The AAV life cycle consists of two phases, the productive or replication phase and the latent phase (Figure 6.3) (Berns, 1990; Carter, 1990). In the productive life cycle, AAV co-infects the host cell with a helper virus (adenovirus or herpesvirus). As the helper virus replicates, AAV replication also occurs, along with encapsidation of progeny virions. These virions are released if and when the helper virus lyses the cell. The helper virus effects are indirect. In some cells under special conditions cellular factors can support AAV replication without helper virus following treatment with genotoxic agents such as ultraviolet (UV) irradiation, gamma irradiation, or hydroxyureas (Schlehofer *et al.*, 1986).

If cells are infected with AAV in the absence of helper virus, AAV enters the latent phase of its life cycle. This generally involves stable integration of tandem head-to-tail dimers of the AAV genome. However, episomal forms of AAV have also been found to persist in chronically infected cells for at least 100 passages (Cheung *et al.*, 1980; Hoggan *et al.*, 1972). The morphology and growth characteristics of cells are not overtly affected by AAV latency. If latently infected cells are subsequently infected with helper virus, AAV can be rescued, i.e. it can re-enter the productive phase of the life cycle.

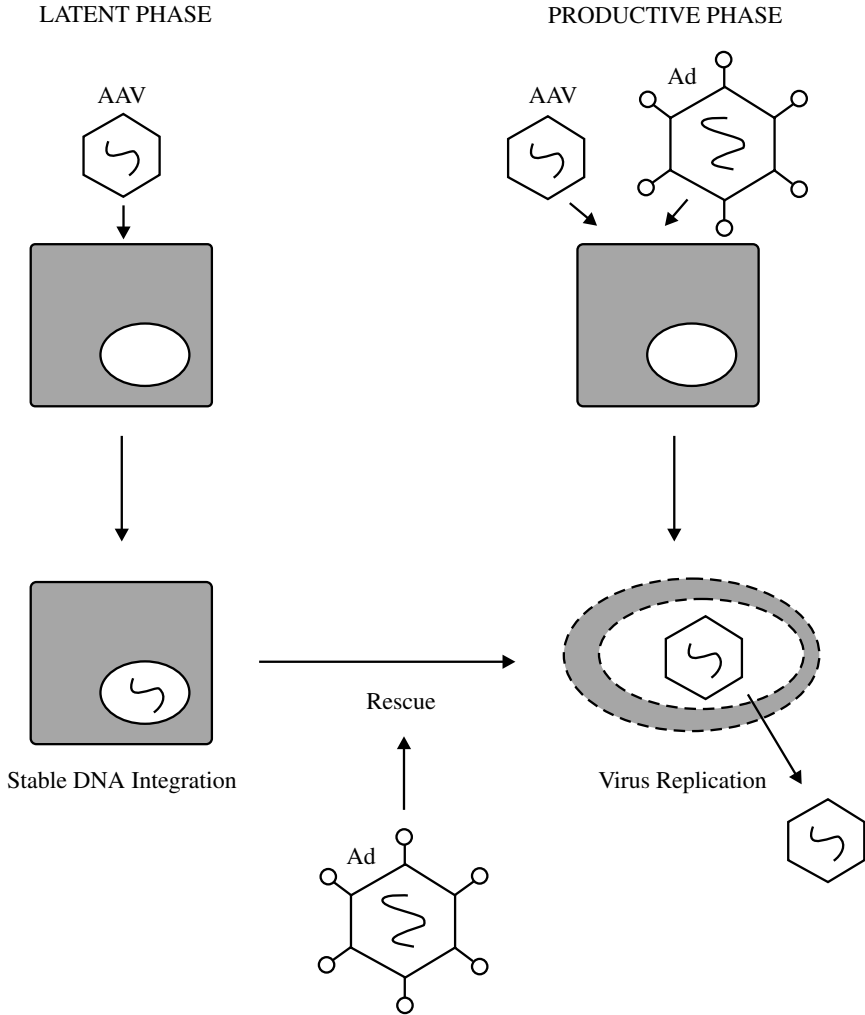


Figure 6.3. AAV life cycle. The latent and productive phases of the AAV life cycle are depicted. See text for details.

6.2.4 SITE-SPECIFICITY OF AAV INTEGRATION

One of the unusual features of AAV latency is the tendency for AAV to integrate within a specific region of human chromosome 19, the AAV-S1 site. In studies of immortalized cell lines infected with AAV, several groups found AAV integrants within the same region of chromosome 19 (q13.3-qter) in approximately 65–70% of cell clones (Kotin *et al.*, 1990, 1991, 1992;

Samulski, 1993; Samulski *et al.*, 1991). This 8.2 kb-AAV-S1 site has been sequenced and found to contain a number of important elements including homologues of the AAV *rrs* and terminal recognition sequence (*trs*). Giraud *et al.* (1994), have demonstrated that a 0.5 kb fragment of the S1 site, when incorporated into an episomal Epstein-Barr virus (EBV) plasmid, is sufficient as a target for AAV integration. The mechanism for integration may involve the Rep68 protein, as recent data from Weitzman *et al.* (1994), indicate. In those studies, Rep68 was found to bind to both the AAV-ITR and the AAV-S1 sequence simultaneously, forming a complex which could serve as a pre-integration intermediate.

6.3 AAV-DERIVED RECOMBINANT VECTORS

6.3.1 STRUCTURE OF RECOMBINANT AAV VECTORS

In an effort to exploit the unique features of the AAV life cycle in a gene transfer vector, several groups constructed recombinant vectors by deleting internal portions of the AAV genome within plasmids and inserting transgenes of interest (Flotte *et al.*, 1992; Hermonat and Muzyczka, 1984; Samulski *et al.*, 1989; Tratschin *et al.*, 1984). In early experiments, AAV vectors contained substantial portions of the *rep* and/or *cap* genes, and were complemented either with wild-type AAV or with overlapping partially deleted constructs. These vectors demonstrated the feasibility of using AAV as a eukaryotic vector, but were limited by the presence of wild-type virus, which appeared to exert transcriptional suppressor effects mediated by the *rep* gene.

Samulski *et al.* (1989), demonstrated that preparations of AAV vectors substantially free of wild-type AAV could be generated if non-overlapping constructs were used (Figure 6.4). In this packaging procedure, vector constructs contained the gene of interest flanked by AAV-ITRs, while the complementing 'packaging' plasmid expressed the AAV *rep* and *cap* genes from a second plasmid co-transfected into adenovirus-infected cells. Since the ITRs contain the packaging signals, any suitably sized vector construct (≤ 5 kb from ITR to ITR) would be packaged, while the complementing *rep/cap* expression construct would not.

6.3.2 STRATEGIES FOR PACKAGING AAV RECOMBINANT VECTORS

In order to encapsidate recombinant AAV vector DNA into infectious virions, five elements are generally required: (i) cells permissive for AAV replication (e.g. 293 cells), (ii) a helper virus (e.g. adenovirus), (iii) a recom-

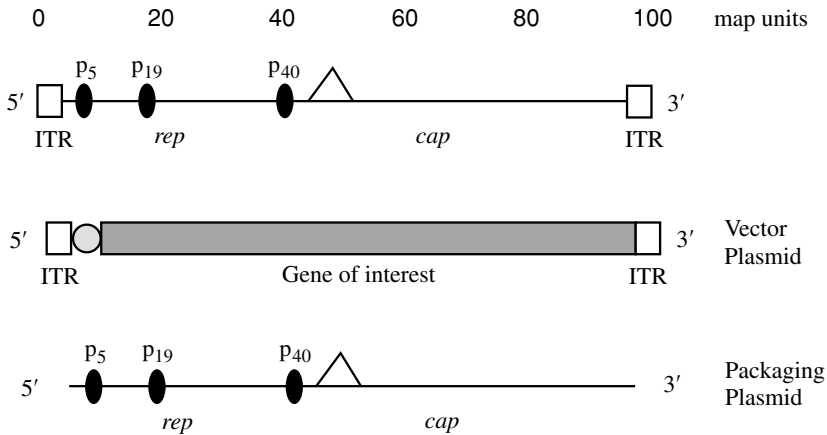


Figure 6.4. Organization of AAV2-based vectors. The AAV genome with a map-unit scale is depicted above, with the ITRs as open boxes and the transcription promoters (P₅, P₁₉, P₄₀) as dark shaded ellipses. Vector plasmids (middle diagram) are constructed by inserting the foreign gene of interest (lightly-shaded bar) and a promoter (lightly-shaded circle) between the ITRs, which serve as replication origins and packaging signals. Vector DNA is packaged into infectious AAV particles after co-transfection with an ITR-deleted packaging plasmid (bottom diagram) into adenovirus-infected cells.

binant AAV vector of 5 kb or less, including ITRs flanking the transgene and any promoter, enhancer, intron, or polyadenylation elements, (iv) a source of Rep proteins, and (v) a source of capsid proteins. These elements can be supplied by co-transfecting adenovirus-infected cells with two plasmids, one containing the recombinant AAV vector DNA (ITR+, *rep*-, *cap*-) and the other containing the complementing AAV genes (ITR-, *rep*+, *cap*+). Cells must then be lysed by serial freeze-thaw or other physical methods to release the packaged virions. Packaged AAV vector can then be separated from cellular debris and helper virus by CsCl gradient ultracentrifugation.

There are a number of potential limitations on the efficiency of packaging from a simple two-plasmid co-transfection technique. First, there is potential inefficiency of transfection, which could be multiplied by having to have two plasmids independently enter the packaging cells. Cell lines have been produced which either contain a stable copy of the vector DNA (in rescuable form) (Flotte *et al.*, 1995), and/or the *rep/cap* expression plasmid (Clark *et al.*, 1995). The level of Rep expression in the target cell is also a potential limiting factor. The fact that Rep68 can downregulate its own expression from the AAV p₅ promoter has led to a strategy in which Rep is expressed from the HIV long terminal repeat (LTR) promoter in 293 cells, where this promoter is constitutively active (Flotte *et al.*, 1995).

6.3.3 PERSISTENCE OF VECTOR DNA IN TARGET CELLS

The use of AAV as a transducing vector is based on the assumption that recombinant AAV retains certain characteristics of wild-type AAV in its ability to establish persistence. In fact, a number of studies have indicated that wild-type-free, *rep*-deleted AAV vectors can mediate stable transgene expression *in vitro* and *in vivo*. Recent evidence indicates, however, that the mechanism of persistence of *rep*-deleted AAV vectors may be different from that of wild-type AAV (Afione *et al.*, 1996; Goodman *et al.*, 1994; Kearns *et al.*, 1994). *In vitro* studies indicate that AAV-CFTR (cystic fibrosis transmembrane conductance regulator) vectors integrate into the AAV-S1 locus much less frequently than wild-type AAV (Kearns *et al.*, 1994). The total number of integration events appears to be decreased, and those integrations which do occur do not appear to share the same preference for chromosome 19. In a related *in vivo* study in rhesus macaques, AAV-CFTR vector DNA persistence in bronchial epithelial cells was once again observed for up to 3 months (Afione *et al.*, 1996). In this instance, there was episomal persistence of double-stranded DNA copies of the vector. These observations support the hypothesis that specific interactions between Rep68, the AAV-ITR, and the AAV-S1 sequence may be involved in site-specific integration by wild-type AAV.

6.3.4 HOST CELL FACTORS AFFECTING AAV VECTOR TRANSDUCTION

The effects of host cell proliferation and helper virus gene expression on AAV vector transduction have also been examined. With regard to cell division, several studies have indicated that vector DNA entry can occur in quiescent cells (Flotte *et al.*, 1994; Podsakoff *et al.*, 1994; Russell *et al.*, 1994). The effects on transgene expression are variable. One study indicated that when cells are infected with vector while quiescent and then allowed to re-enter the cell cycle to undergo *neo*-selection, there was little change in transduction efficiency as compared with proliferating cells (Podsakoff *et al.*, 1994). In another study, it was found that a much higher multiplicity of infection was required in slowly dividing cells in order to achieve a similar level of transgene expression as compared with rapidly proliferating cells (Flotte *et al.*, 1994). In a third study, there was a marked decrease in transduction efficiency in quiescent cells as compared with proliferating cells, but only one multiplicity of infection was used (Russell *et al.*, 1994). Related studies have indicated differences in transduction efficiency between immortalized and primary cells (Halbert *et al.*, 1995), although several other studies *in vitro* and *in vivo* found no such differences (Kaplitt *et al.*, 1994). Finally,

enhancement of expression by UV irradiation has recently been described (Alexander *et al.*, 1994).

Another recent study has indicated that AAV vector expression can be enhanced by concurrent adenovirus infection or expression of the adenovirus E4-orf6 gene product (Fisher *et al.*, 1996). There is a suggestion that this effect is mediated by enhancement of leading strand synthesis in the conversion of the single-stranded DNA to a double-stranded DNA version of the vector. Unfortunately, this study did not examine the role of multiplicity of infection or the kinetics of leading strand synthesis in the absence of Ad E4-orf6 expression.

6.3.5 SUMMARY OF ADVANTAGES AND DISADVANTAGES OF AAV TRANSDUCING VECTORS

The potential advantages and disadvantages of current AAV-based vectors for gene therapy are summarized in Table 6.1. The principal advantage of AAV over other DNA virus vectors, such as adenovirus, is the lack of any viral coding sequence within the vectors, which prevents transduced cells from being recognized and rejected by the immune system. The AAV virion itself also appears to be less pro-inflammatory on initial exposure than the adenovirus virion. These two factors probably account for the very favorable safety profile of AAV vectors in animals (Conrad *et al.*, 1994; Flotte *et al.*, 1993b). Recombinant AAV is also efficient at cell entry, and tends to persist in cells over long periods of time. The principal disadvantages of AAV relate to the fact that it enters the cell as single-stranded DNA and must be converted to double-stranded DNA prior to expression of the transgene. This may limit the level of expression in some cells. Also, as mentioned above, AAV vectors which lack the *rep* gene do not appear to integrate at as high a frequency as wild-type AAV. This could ultimately limit the duration of expression. The packaging limit of AAV is relatively small, at 5 kb. Since vectors must contain the ITRs (0.3 kb total), this leaves only 4.7 kb for the entire insert, including the transgene coding region, the promoter, the polyadenylation signal, and any other regulatory elements. Although AAV vector production is still a relatively inefficient process, this will benefit from further refinements to allow potential widespread clinical use of these agents.

6.4 APPLICATIONS OF AAV VECTORS

Table 6.2 summarizes some of the published applications of AAV gene transfer vectors to specific cell targets or disease models. These include both *in vitro* and *in vivo* experiments.

Table 6.1 Potential advantages and disadvantages of AAV vectors for gene therapy

Advantages	Disadvantages
1. Non-immunogenic (no viral coding sequences)	1. Requires conversion to double-stranded DNA (may delay expression)
2. No host inflammatory reaction to capsid components	2. Decreased integration frequency in absence of Rep proteins
3. Efficient entry of DNA into target cell	3. Small packaging limit (4.7 kb insert)
4. Long-term DNA persistence in target cell	

Table 6.2. Applications of AAV vectors

<i>In vitro</i>		<i>In vivo</i>	
Cell targets	Disease models	Cell targets	Disease models
K562 (erythroid)	Thalassemias	Rabbit bronchus	Cystic fibrosis
CD34+ PBLs	Sickle cell disease	Rhesus bronchus	Cystic fibrosis
Murine bone marrow cells	Hemoglobinopathies	Rat brain	Parkinson's disease
IB3-1 (bronchial)	Cystic fibrosis		
Human fibroblasts	Gaucher's disease and metachromatic leukodystrophy		
CD4+ T lymphocyte lines	AIDS		
Immortalized B lymphocytes	Chronic granulomatous disease		

6.4.1 *IN VITRO* APPLICATIONS

AAV transducing vectors were initially studied in immortalized cell lines, such as HeLa, 293, and KB, using reporter genes, such as neomycin phosphotransferase (*neo*) and chloramphenicol acetyltransferase (CAT) (Hermonat and Muzyczka, 1984; Tratschin *et al.*, 1984, 1985). The utility of these vectors has been confirmed in more differentiated cell lines, including the K562 erythroleukemia cell line (Walsh *et al.*, 1992), the IB3-1 cystic fibrosis (CF) bronchial epithelial cell line (Flotte *et al.*, 1992), and several CD4+ lymphoid cell lines (Chatterjee *et al.*, 1992). AAV vectors have also been used in primary cell types and in *in vitro* models of specific disease applications.

A number of studies have focused on the use of AAV vectors in bone marrow-derived cells as a potential treatment for hemoglobinopathies. Walsh *et al.* (1992), initially demonstrated regulated high-level expression of a human γ globin gene in the K562 erythroleukemia cell line. Subsequently, the same group demonstrated the feasibility of using their AAV-globin vector to transduce CD34+ progenitor cells derived from the peripheral blood of a patient with sickle cell anemia (Miller *et al.*, 1994). Similar results were obtained with CD34+ cells from rhesus monkeys and humans infected with AAV- β -galactosidase vectors or wild-type AAV (Goodman *et al.*, 1994). AAV vectors have also been used to express antisense to the α globin gene, which could have therapeutic effects in β thalassemia, where an imbalance of α and β globin contributes to abnormal erythroid maturation (Ponnazhagan *et al.*, 1994).

AAV vectors have also been used in other models of primary hematopoietic disease. AAV reporter vectors have been used in murine hematopoietic precursors (Zhou *et al.*, 1993). Another important study utilized cells from a patient with Fanconi's anemia, complementation group C (FACC) (Walsh *et al.*, 1994). Transduction of progenitor cells with an AAV-FACC vector resulted in phenotypic correction of the basic defect in DNA repair and restoration of the colony forming ability which is deficient in this disease. AAV vectors have also been used to transfer the NADPH-oxidase gene, which is deficient in chronic granulomatous disease (Thrasher *et al.*, 1995), the glucocerebrosidase gene, which is deficient in Gaucher's disease, and the arylsulfatase A gene, which is deficient in metachromatic leukodystrophy (Wei *et al.*, 1994). Another report described the use of an AAV antisense vector for inhibition of HIV-1 replication in lymphoid cell lines as a potential treatment for the acquired immunodeficiency syndrome (AIDS) (Chatterjee *et al.*, 1992).

Several studies have examined the potential utility of AAV as a gene transfer vector for CF. AAV vectors for expressing the CFTR were constructed using small endogenous AAV promoter elements in order to facilitate packaging of the 4.5 kb CFTR coding region, which along with the mandatory 0.3 kb for the ITRs produces a vector size near the packaging limit of AAV (Flotte *et al.*, 1993a). The AAV-CFTR vector constructs produced were able to be packaged and were used to transduce the CF-defective IB3-1 cell line, resulting in phenotypic correction of the chloride transport defect. Interestingly, CFTR expression resulted in both the appearance of a small linear chloride conductance associated with recombinant CFTR expression and the restoration of cAMP-responsiveness of the outwardly-rectifying chloride channel (Egan *et al.*, 1992; Schwiebert *et al.*, 1994). The demonstration of phenotypic correction of a bulk culture of cells without selection encouraged pursuit of further *in vivo* studies of AAV-CFTR gene transfer.

6.4.2 *IN VIVO* APPLICATIONS

Published reports of *in vivo* gene transfer with AAV vectors have focused on the brain and the lung as potential target organs. Kaplitt *et al.* (1994) demonstrated that AAV vectors expressed the *lacZ* gene for over 3 months after direct injection into the rat brain. They also showed that an AAV vector expressing the tyrosine hydroxylase (TH) gene effected a partial phenotypic correction in a rat model of Parkinson's disease. AAV-CFTR vectors have been studied in the lungs of rabbits (Flotte *et al.*, 1993a) and rhesus monkeys (Afione *et al.*, 1996) after delivery via fiberoptic bronchoscopy. In each case, long-term vector DNA persistence and RNA expression were observed without overt toxicity. These studies have served as a basis for a phase I clinical trial of AAV-CFTR administration to the nose and lung of adult CF patients with mild lung disease which has recently been initiated (Flotte *et al.*, 1996).

6.5 SAFETY ISSUES

AAV is based on a virus which commonly infects humans without causing disease, and AAV vectors have had a remarkably favorable safety profile in *in vivo* tests. Nevertheless, it is important to consider potential safety issues to be assessed in future preclinical and clinical trials. Safety issues with AAV vectors may be considered in terms of (i) potential risks to the intended recipient of the gene therapy vector, i.e. the subject, and (ii) potential risks associated with spread of the recombinant virus to other individuals, i.e. environmental contacts.

6.5.1 SUBJECT SAFETY

Recent *in vivo* data suggests that there is no vector-related toxicity associated with AAV vector administration to the lungs of rabbits and rhesus monkeys or to the brains of rats. Therefore, safety concerns with AAV vectors remain largely theoretical, and are based on the experience with other viral vectors whose biological characteristics differ substantially from those of AAV.

The possibility of insertional mutagenesis and subsequent tumorigenesis was considered because *rep*-deleted AAV vectors have been found to integrate non-specifically into some cells within the target population. However, DeLaMaza and Carter (1981) examined the tumorigenic potential of both *rep*⁺ and *rep*⁻ AAV in a newborn hamster model of tumorigenesis. There was no evidence of enhanced tumorigenesis with either virus. In fact, both *rep*⁺ and *rep*⁻ AAV were found to suppress the tumorigenic potency of adenovirus type 12. Furthermore, there has been no evidence of neoplastic changes on long-term follow-up of the animals involved in the *in vivo* gene

transfer studies mentioned above (Flotte *et al.*, 1993a; Kaplitt *et al.*, 1994). Based on this experience, the mutagenesis risk from these vectors appears to be low.

The possibility of vector-induced inflammation and cell-mediated immune responses was raised because adenovirus vector administration to the lung is associated with both a dose-related inflammatory response (Simon *et al.*, 1993) and subsequent immune-mediated elimination of transduced cells (Yang *et al.*, 1994). These issues have been directly studied with AAV vectors in the rhesus macaque (Conrad *et al.*, 1994). Bronchoscopic delivery of AAV-CFTR to the bronchial epithelium was not associated with any detectable inflammation as judged by bronchoalveolar lavage fluid analysis (including cell counts, and interleukin-6 (IL-6) and interleukin-8 (IL-8) levels), radiographic studies, pulmonary function studies, and histopathological examination. These studies included doses of vector as high as 1×10^{11} total particles and time points ranging from 10 to 180 days. The two key differences between AAV and Ad in this regard would seem to be: (i) that AAV capsid proteins have less cytotoxicity and pro-inflammatory effects and (ii) that the absence of any viral coding sequences within AAV vectors prevents transduced cells from becoming targets for cellular immune surveillance.

6.5.2 ENVIRONMENTAL SAFETY

Until it is established in clinical trials that AAV vectors are safe in humans, it is important to prevent the exposure of individuals other than the subjects themselves. For *ex vivo* manipulations, standard biosafety level 1 precautions have been sufficient for this agent. There are additional issues related to *in vivo* gene transfer. These include: (i) shedding of recombinant AAV from individuals immediately after vector administration, and (ii) rescue or subsequent shedding from vector-treated individuals who may be later infected with wild-type AAV and adenovirus. Each of these issues was studied in the rhesus macaque model (Afione *et al.*, 1996). Shedding after initial exposure was found to be undetectable by 3 days in the nasal fluid, bronchial lavage, urine, and stool. Rescue was studied in several different ways, varying the site and sequence of administration of Ad, wt-AAV, and AAV-CFTR vector. In most instances, no subsequent vector shedding was observed. When a large inoculum (10^{10} total particles) of wild-type AAV and then AAV-CFTR were administered to the same site in the lower respiratory tract, followed by Ad infection, a very low level of AAV-CFTR shedding was detectable in the lung, which lasted for 6 days. These findings indicate that the risk of environmental exposure will be low. Shedding studies are currently under way as part of phase I trials of AAV-CFTR administration.

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7 Advances in Engineering HSV Vectors for Gene Transfer to the Nervous System

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7.1 INTRODUCTION

Gene therapy offers a potentially promising approach to the treatment of central nervous system (CNS) disorders where the blood–brain barrier prevents access by systemically delivered pharmacologic agents. Moreover, the complex cellular and regional specialization of the brain can require drug delivery to specific brain regions and component cell types. Neurologic diseases that are currently considered amenable to therapeutic intervention by gene therapy include neurodegenerative disorders, brain tumors, and autoimmune syndromes that lead to the destruction of nerve tissue components. The progression of chronic neurodegenerative disorders such as Alzheimer's disease and Parkinson's disease might be ameliorated by the local production of neurotropic factor(s) that prevent the degeneration of hippocampal neurons or dopaminergic neurons of the nigral-striatal bundle, respectively. Since experiments in animal models have shown that disease progression may be prevented by specific neurotrophins (e.g. nerve growth factor (NGF) or glial-derived neurotrophic factor (GDNF) [1–4] that act focally, these applications will require a vector that can constitutively express low levels of the neurotrophin in specific cells within the region of interest for the life of the host. Brain tumors represent a second class of neurologic disease that might be treated by transient, localized expression of therapeutic molecules such as immune modulators that attract tumor-killing inflammatory cells and tumor-specific immune T cells, cytotoxic molecules such as tumor necrosis factor, or enzymes such as thymidine kinase (TK) that will locally activate anti-cancer drugs. Activated pro-drugs such as ganciclovir and 5-fluorouracil can also kill neighboring dividing cells by cell-to-cell trans-

mission in the first case or uptake of locally released drug in the second. Multiple sclerosis, a progressive demyelination caused by immune-mediated attack, represents an autoimmune disease that might be effectively treated by the local expression of immunomodulatory cytokines or cytokine inhibitors.

Therapeutic genes may be introduced into relevant cells of the nervous system by direct gene transfer *in vivo* or through an *ex vivo* approach involving transplantation of transduced cells. The *ex vivo* approach consists of introducing the therapeutic gene into a cell population such as fetal or immortalized neurons, multipotent progenitor neural stem cells, adrenal chromaffin cells, glia, or fibroblasts, which are then cultured *in vitro*, transplanted back into the host, and survive at or migrate to the relevant anatomic location. *In vivo* gene transfer approaches require vectors capable of delivering the therapeutic gene directly into cells of the nervous system. The major issues in the development of therapeutic vectors for *in vivo* use concern targeting and delivery to the relevant cells, potential vector toxicity or instability, the possibility of host immune responses that either prevent vector entry or eliminate vector-transduced cells, and the level and duration of therapeutic transgenic gene expression.

A wide variety of different delivery vehicles have been tested for gene delivery to neurons and glia, including viral vectors, gold particle-DNA conjugate bombardment, direct injection of plasmid DNA, and cationic liposomes alone or accompanied by fusion-promoting agents. Viral vectors have proved to be an efficient means of gene delivery. Replication-defective genomic herpes simplex virus (HSV) [5–18], HSV amplicon [19–25], adenovirus (AV) [1,26–32], adeno-associated viruses (AAV) [33–36], and human lentivirus-mouse oncovirus recombinant (HIV-MoMuLV) derived [37–41] vectors have all been tested in neurons in culture and in animals. These base viruses differ with respect to tropism, persistence as an episome versus integration into host chromosome, toxicity or antigenicity, longevity and level of gene expression, risk of inducing malignant formation, maximum transduction capacity, and the ability to produce high-titer viral stocks free of replicating virus contaminants. Because HSV naturally establishes long-term latency in neurons of the CNS and parasympathetic nervous system (PNS), a state in which complete shut-off of lytic cycle gene expression enables the virus to evade immune surveillance while continuing to produce a series of latency-associated transcripts (LATs), it should be possible to engineer HSV genomic vectors that are incapable of replicating or expressing cytotoxic and antigenic viral gene products while exploiting the natural LAT promoter elements for transgene expression as an extension of the natural biology of this virus.

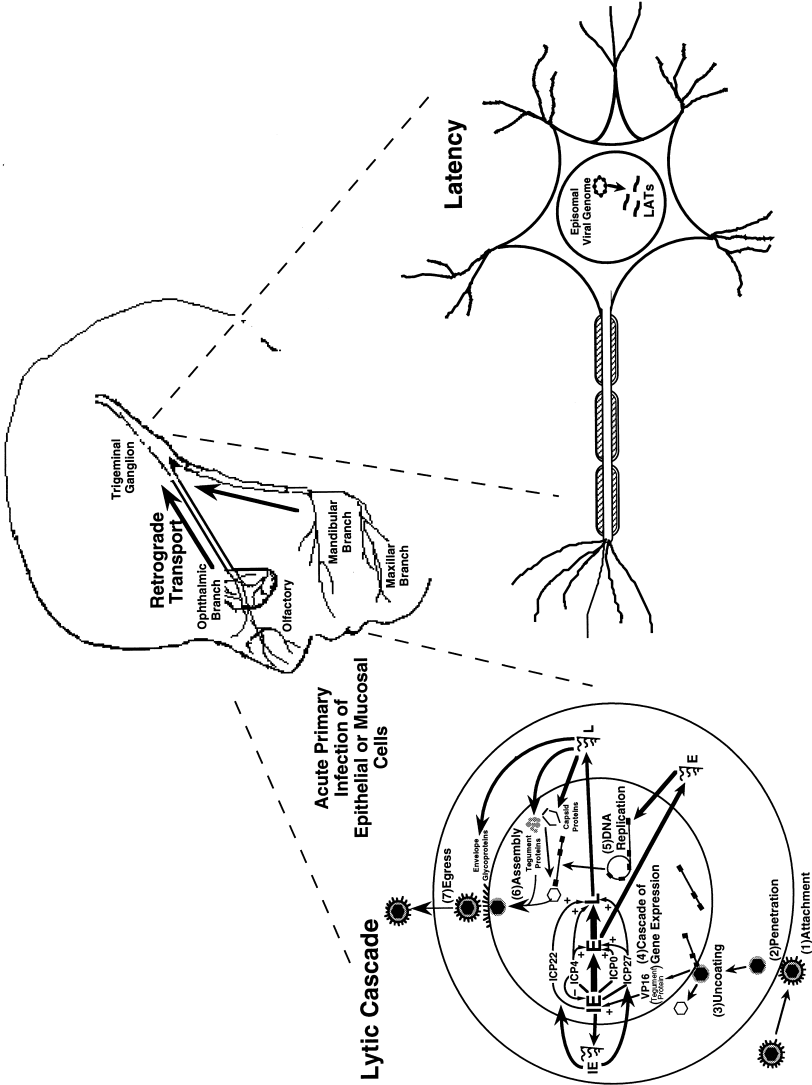
7.2 HSV BIOLOGY

HSV-1 is a commonly acquired, naturally neurotropic virus that establishes a life-long, mostly benign, association with the human host. The virus replicates within epithelial cells of the cornea or orofacial tissue [42,43], invades local peripheral nerve endings, and ascends to the associated sensory ganglion by retrograde axonal transport (Figure 7.1). HSV is capable of maintaining multiple copies of viral genomes as quiescent episomes within post-mitotic sensory neurons of the peripheral nervous system [44–46]. The HSV virion contains the 152 kb double-stranded DNA genome packaged in the shape of a torus within an icosadeltahedral capsid [47]. The genome consists of two unique segments (U_L and U_S) each flanked by a set of terminal and internal repeats. Surrounding the capsid is the tegument, an amorphous mass of proteins containing at least six viral proteins, including the virion host shut-off (vhs) protein that downregulates host cell protein synthesis [48–58], and VP16 or α -transinducing factor (α -TIF) [59–64], a protein which interacts with the cellular factors Oct-1 and HCF [36,65–73] to transactivate the immediate-early (IE) class of viral genes [60–64,69,74–76]. These proteins enter the nucleus with the virus and participate in the infection process. Infectious virions possess an envelope which is acquired by budding of the particle through the inner nuclear membrane. The envelope contains at least ten virus-encoded glycoproteins integrated into the lipid bilayer envelope, which are involved in the attachment, penetration, and cell-to-cell spread of HSV in a variety of different cell types [77–79].

A notable feature of the viral lytic cycle is the temporally and sequentially coordinated cascade of viral gene expression [80]. α -TIF in the virus tegument induces expression of the first kinetic class of viral genes, termed IE or α genes [60–64,69,74–76]. Four of the five α genes (ICP0, ICP4, ICP22, and ICP27) are involved in transcriptional and post-transcriptional regulation of the next kinetic class of viral genes, designated as early or β genes [81–91]. The β genes provide enzymes required for nucleotide metabolism and viral DNA replication. The last kinetic class of viral genes to be expressed, the γ genes, require viral DNA replication [92,93] as well as the IE gene products for expression. The late genes encode protein products which comprise components of the viral icosadeltahedral capsid, tegument, and envelope glycoproteins.

A similar cascade of viral gene expression occurs within nerve cell bodies in sensory ganglia [94–97] and infectious viral particles can be detected up to 7 days following infection. However, in neurons viral lytic gene expression may be repressed by an unknown mechanism, following which viral genomes are maintained as non-replicating, largely quiescent nucleosome-bound episomes within the nucleus [44–46,98]. The latent genomes may be

Figure 7.1. HSV-1 infection of the host. Primary HSV-1 infection of epithelial or mucosal cells of orofacial region of the host results in a lytic infection yielding progeny virus particles that encounter axonal termini that innervate the site of primary infection. Retrograde axonal transport of the virus back to nerve cell bodies within the trigeminal ganglion can either lead to lytic replication or, usually, result in the establishment of a 'quiescent' or latent infection. During latency the viral genome remains as a circular episome that is transcriptionally silent except for the expression of the latency-associated transcripts (LATs). The virus can be stimulated to reactivate from the latency, enter the lytic cycle and produce progeny virions which reach the periphery by anterograde axonal transport, where recurrent infection may lead to a visible lesion.



induced to reactivate by stimuli such as stress and exposure to ultraviolet (UV) irradiation. Upon reactivation, viral nucleocapsids are transported back to the periphery, where acute replication resumes within epithelial cells. In humans, manifestations of viral reactivation include characteristic cold sores or herpetic keratitis, depending on the site of recurrence. Thus, the virus may oscillate between two states in the host: long periods of dormancy or 'latency,' interrupted by occasional acute periods of active viral replication, or in some individuals the virus may remain dormant for the life of the host without recurrent outbreaks.

During latency, viral transcription is limited to a unique set of transcripts originating from a 10 kb region located in the internal (IR_L) and terminal (TR_L) repeats of the viral genome (Figure 7.2). Two transcripts, 2.0 and 1.5 kb in length, accumulate in latently infected sensory ganglia and are referred to as latency-associated transcripts (LATs) [99–106]. Recent evidence [107–109] suggests that the 2.0 and 1.5 kb LATs are stable introns derived from an unstable 8.3 kb primary transcript [110], a result that is consistent with the nuclear localization, lack of polyadenylation, and non-linear nature of the LATs [107–109,111,112].

The functional role of the LATs is not known. Deletions in the LAT region do not have deleterious effects on the ability of the virus to replicate or to establish latency [113–124] but do appear to delay or reduce the ability of the virus to reactivate [117,118,121,124–132]. Recent work using trigeminal ganglion cells suggests that LAT functions to increase the number of neurons in which the virus can persist [133]. Because mutations that affect LAT expression have not been shown to prevent the establishment of latency, it should be possible to exploit the LAT promoter-regulatory region to drive latency-specific expression of a therapeutic gene inserted in place of LAT.

7.3 DEVELOPMENT OF HSV VECTORS FOR THE NERVOUS SYSTEM

In engineering viral vectors, one must consider the natural tissue tropism, episomal versus integrated state of the viral vector, degree of toxicity and/or antigenicity, longevity and desired level of gene expression, risk of tumorigenicity, maximum transduction capacity, and in some cases the ability to produce high-titer viral stocks free of replicating virus contaminants. The natural neurotropism of HSV and persistence of its genome as episomes within target cells are two natural features of the virus that make it an attractive candidate vector for gene therapy of the nervous system. This section summarizes progress made in engineering HSV vectors for therapeutic intervention in the nervous system using recombinant viruses that address some of the other variables listed above.

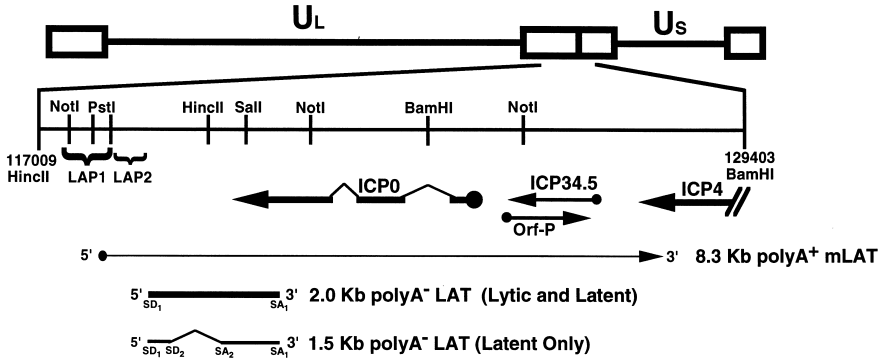


Figure 7.2. HSV latency gene expression. The location of the various HSV latency-associated transcripts (LATs) relative to the ICP0, ICP34.5, Orf-P, and ICP4 lytic gene RNAs within the prototype genome is depicted. The latency active promoters LAP1 and LAP2 are shown relative to the 5' end of the 8.3 kb mLAT and the 2.0 kb LAT intron. The 2.0 kb LAT intron is derived from the 8.3 kb mLAT using the indicated splice donor/acceptor (SD₁/SA₁) splicing signals during lytic and latent infection. The 1.5 kb LAT is derived by the splicing of the 0.5 kb LAT twintron from the 2.0 kb LAT using the SD₂/SA₂ splicing signals exclusively during latency.

7.3.1 DELETION OF ACCESSORY FUNCTIONS TO INCREASE THE CAPACITY FOR FOREIGN GENES

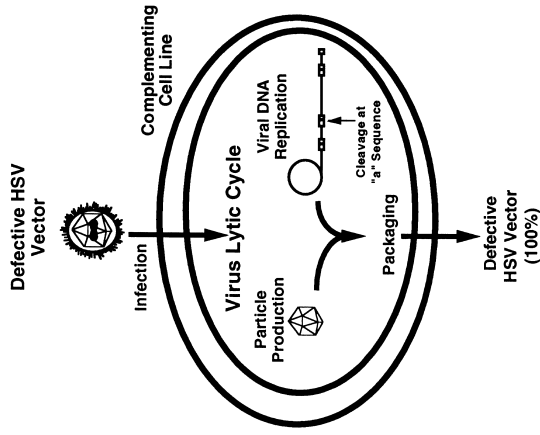
The HSV genome contains 84 known open reading frames [134], many of which are complemented by host cell proteins or provide accessory functions for viral replication and spread in the host. This made it possible to construct viral recombinants wherein non-essential genes of HSV-1 were replaced with transgene expression cassettes without affecting the ability of the virus to replicate in culture. Vectors mutated in particular non-essential accessory functions were capable of replicating in dividing cells (e.g. tumor cells) but not in post-mitotic cells (e.g. brain neurons). For instance, viruses deleted for genes encoding DNA synthesis enzymes (e.g. TK or ribonucleotide reductase) or the gene involved in neurovirulence (e.g. ICP34.5) are compromised for growth in post-mitotic neurons in the brain [135–137], and thus showed reduced pathogenicity upon intracranial inoculation. Although these vectors were still highly toxic to cells in culture and displayed reduced cytotoxicity *in vivo*, they have still been employed as oncolytic agents in treatment of gliomas since the actively dividing nature of the tumor cells provides an adequate supply of nucleotide pools and DNA synthesis machinery for viral replication, and thereafter, tumor cell lysis [138–146]. However, these viruses are extremely toxic to dividing cells in culture and a majority of cell types *in vivo*, and thus their utility in most gene therapy applications is limited.

Non-essential genes can be individually deleted from the viral genome without preventing virus replication under permissive tissue culture conditions used for culturing virus [147,148], although some deletion mutants grow less vigorously than wild-type virus and the removal of particular accessory genes can significantly impair replication. The U_S segment of HSV-1 contains 13 genes of which only one is required for virus growth in tissue culture (U_{S6} encodes gD). Deletion of this DNA segment followed by rescue of the unique essential gene within a U_L locus creates genomic space (~ 20 kb) for the insertion of either large or multigene cassettes. In addition, the U_S segment encodes four other non-essential glycoproteins (gJ, gG, gI, and gE) whose deletion will eliminate their potential contributions in virus attachment as well as to the host immune response to the virus envelope. Deletion of U_{S1} through U_{S5} along with part of the IR_S repeat resulted in a recombinant that displayed reduced pathogenesis *in vivo* [149]. However, much of the U_S segment still remains in this recombinant. Recently we engineered a recombinant which deleted 8.5 kb from the U_S segment covering U_{S3} to the U_{S11} in a mutant in which the gD gene was then used to replace gC [150]. This virus displayed a complete abrogation of cell-to-cell spread *in vitro* and failed to effectively spread to the PNS following topical corneal inoculation. Moreover, deletion of the U_S segment coding for U_{S3} to U_{S11} may also reduce immune recognition of the vector by eliminating potential anti-viral target antigens while increasing the transduction capacity of the vector.

7.3.2 VECTOR-ASSOCIATED CYTOTOXICITY

Although the first-generation genomic HSV vectors (Figure 7.3A) deleted for ICP4 were able to efficiently transduce cells in culture and neurons *in vivo*, they proved to be highly toxic for both primary neurons and muscle cells as well as other non-permissive dividing cells in culture, even at low multiplicities of infection (MOI) [151–154]. It is presumed that expression of the remaining HSV IE gene products, which continue to be expressed in ICP4⁻ mutants, that have transcriptional modifying activities (ICP0, ICP22, ICP27) account for this toxicity, since both early and late gene expression are extremely reduced or eliminated with the ICP4 deletion mutants [81,155], and interferon treatment which disrupts IE gene expression [156–158] reduces the toxicity of the virus [159]. UV-inactivated virus shows markedly reduced cytotoxicity in myogenic [160] and other cells [161,162] in culture. The fourth IE gene which is overexpressed in the absence of ICP4 is ICP47. ICP47 is not likely to be cytotoxic since the activity of ICP47 involves assisting the virus in evading the host immune system by interfering with major histocompatibility complex (MHC) class I antigen presentation [163–166]. Another potential cytotoxic gene is U_{L41} , which codes for a tegument

(A) Defective HSV Vector



(B) Amplicon System

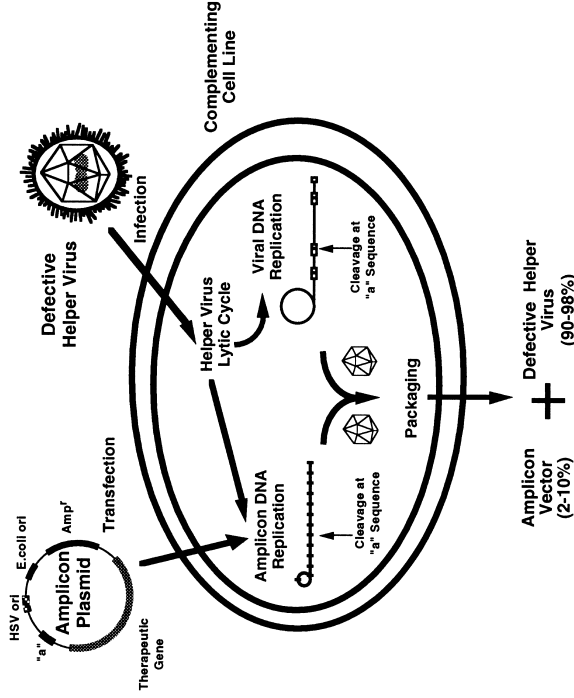
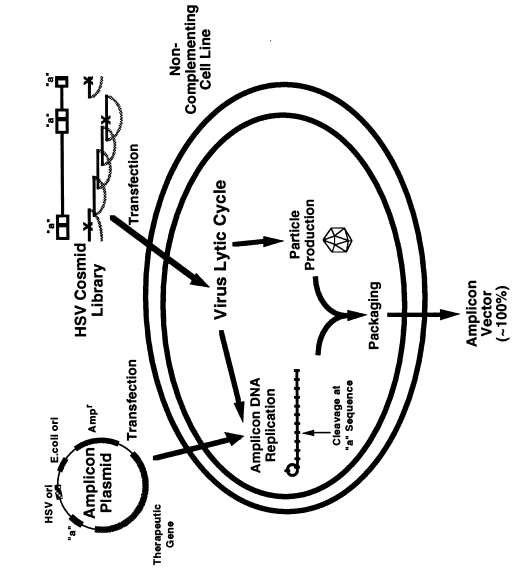
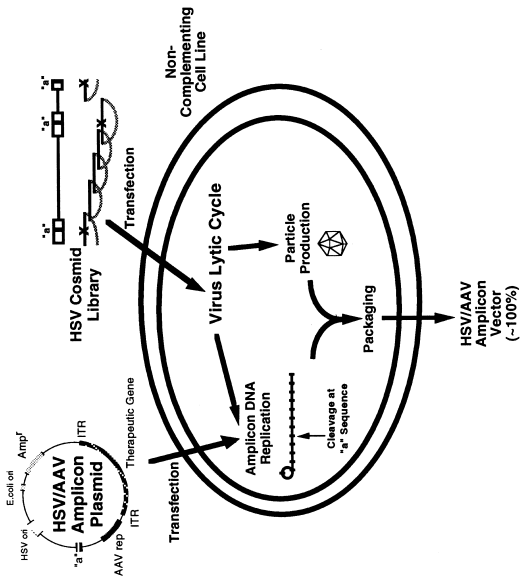


Figure 7.3. HSV vector strategies. (A) Production of defective genomic HSV vectors is carried out in cell lines that are engineered to provide the deleted essential IE genes *in trans*. These vectors are incapable of replicating in neurons because of the missing essential IE genes. (B) Amplicons are propagated in bacteria using the bacterial origin of replication (*E. coli* ori), and then transfected into a complementary cell line that is infected with defective 'helper' HSV. The amplicon plasmid is subsequently propagated due to the presence of an HSV origin (HSV ori) and packaging signal ('a' sequence) and incorporated into particles consisting either of amplicon concatamers (about 150 kb in length) or defective HSV genomes. (C) The helper virus-free amplicon system does not require either a defective helper virus or a complementing cell line for the amplicon plasmid to be packaged. The amplicon plasmid

(C) Helper Virus-Free Amplicon System



(D) HSV/AAV Hybrid System



is transfected into cells along with five cosmid containing overlapping fragments that represent the entire HSV genome, which encode all the viral proteins necessary to produce infectious particles. In order to insure that only the amplicon plasmid is packaged and not any of the cosmid, the packaging signal ('a' sequence) was deleted from the cosmid clones. (D) The hybrid HSV-AAV system uses the helper virus-free amplicon system to package an amplicon that contains the therapeutic gene flanked by the AAV terminal repeats (ITRs) and in addition encodes the AAV rep gene, which enables the integration of the therapeutic gene into the host chromosome when rep is expressed.

protein (vhs) that is responsible for shut-off and destabilization of host cell mRNA [52,53,55–57,167]. Deletion of any of the four IE genes alone or deletion of vhs did not reduce toxicity [159]. However, ICP4, ICP0, ICP22, and ICP27 were all shown to be toxic to cells in stable transfections assays [161], suggesting that ICP0, ICP22, and ICP27 contribute to the toxicity associated with the first-generation ICP4-deficient recombinant vectors. Thus, it may be necessary to delete all four of the IE transactivating gene products in combination in order to eliminate vector-mediated toxicity, providing a vector capable of gene delivery to cells at high multiplicity of infection without cell death.

The safest vector would be deleted for the viral IE genes that are required for activation of early and late viral functions *in vitro*. Multiply deleted viral mutants have been developed that are incapable of replicating in neurons or any cells other than their stably transformed complementing cell lines. Mutants deleted for both the ICP4 and ICP27 IE gene functions [151–153] were less cytotoxic than either single gene deletion mutant alone. However, they still displayed residual toxicity [151,152,154]. Deletion of ICP22 from the background of an ICP4⁻/ICP27⁻ virus significantly diminished vector-associated toxicity [154]. Mutants deleted for these genes are capable of transducing some tissue culture cells without causing cell death at multiplicities of infection of 10 or less. However, ICP0 is overexpressed in this mutant background and can interfere with cellular growth, genome replication, and cell division [154]. Although removal of ICP0 in addition to ICP4 and ICP27 further reduced toxicity and significantly prolonged the length of transgene expression *in vitro* [168], the level of gene expression is considerably reduced compared to a vector lacking just ICP4, ICP22, and ICP27. Systematic removal of these genes is intended to create a panel of viral vectors that can be selected based on the degree of viral cytotoxicity and viral antigen production as well as optimal transgene expression *in vivo*. Vector-associated antigenicity is especially a problem in the immune competent host where immunologic memory would activate effector T cells, which can then eliminate the vector-containing cells. Finally, such vectors are essentially incapable of reactivating from latency, thus providing a relatively safe, gene transfer vector with a natural propensity for the nervous system that may also prove useful in therapeutic applications to other tissues.

7.3.3 PROMOTER SYSTEMS FOR TRANSGENE EXPRESSION

The longevity and level of gene expression required of a gene transfer vector depend on the nature of the therapeutic application. Long-term, low-level therapeutic gene expression may be required to treat chronic or progressive degenerative disorders such as Parkinson's disease or Alzheimer's disease, while high-level but transient production of therapeutic protein may be

suitable for stemming tissue damage during acute neurological events such as stroke or seizure. Neuronal-specific cellular and viral promoters have been employed in order to achieve appropriate levels of transgene expression in target populations, while inducible expression systems have been developed to affect the timing of transgene expression.

7.3.3.1 Transient Expression

A variety of promoters have been employed to drive reporter/therapeutic gene expression from first-generation genomic and amplicon HSV vectors in the nervous system. Numerous HSV lytic cycle promoters, including α (IE) gene promoters ICP0 [10,12,169–171] and ICP4 [7,12,169,172,173], the α/β (IE/E) gene promoter ICP6 [12,14,136,142,169,172,174–176], β (E) gene promoters ICP8 [172,177] and TK [178], and γ (L) gene promoters such as gC [135,171,179] have been employed in genomic vectors. However, in all instances they remain active only during acute infection. Although HSV α (IE) gene promoters have been used extensively in the amplicon system [19–15,180–195] to achieve prolonged transgene expression, this activity was potentially influenced by the presence of helper virus in the amplicon preparations or recombination between the amplicon plasmid and the helper virus genome [187,188,196].

Promoters from other viruses such as SV40 [17,194,197–199], JC virus [194], human cytomegalovirus (HCMV) [8,10,11,14,17,142,197,200–207], or the long terminal repeats (LTRs) from HIV [208], Rous sarcoma virus (RSV) [209], and Moloney murine leukemia virus (MoMLV) [6,9,10,15,177,210,211] have all yielded transient transgene expression in the nervous system or other tissues except in instances when the MoMLV LTR was juxtaposed to the HSV latency-active promoters [8,210–212].

Cellular promoters such as β globin [213–215] or albumin [216] as well as the nerve-specific enolase (NSE) [5,177,217, Bender and Glorioso, unpublished], neurofilament (NF) [210,218, Bender and Glorioso, unpublished], or the hypoxanthine phosphoribosyltransferase (HPRT) [Lee and Glorioso, unpublished] neuronal-specific promoters failed to display cell-specific activity or remain active in the background of HSV genomic vectors. However, either the tyrosine hydroxylase (TH) or proenkephalin neuronal-specific promoters have displayed extended expression within amplicon vectors in CNS applications [181,219–221] and have shown cell-specificity [222]. All of the viral and cellular promoters tested have been from genes transcribed by RNA polymerase II (RNAPII), yet both RNAPI [223] and RNAPIII [224] promoters have been shown to be only transiently transcribed from genomic HSV vectors, suggesting that the transient nature of promoter activity in the first-generation vectors is not based on the class of RNA polymerase involved in the transcription complex.

Expression from the HSV ICP0 and the HCMV IE promoters in neurons in culture and in rodent CNS *in vivo* was prolonged (6–8 weeks post-inoculation) in the context of a third-generation mutant vector background deleted for multiple IE genes (ICP4, ICP27, and ICP22) [225], suggesting that one of the IE gene products deleted in this vector caused the attenuation of reporter gene expression from previous first-generation single IE deletion viral vectors. Neuronal-specific promoters which displayed transient activity in the first generation ICP4-deficient vectors may prove to show nerve cell specificity in the background of the third generation multiple IE gene deletion vectors, and may yield long-term expression as seen in amplicon vectors.

7.3.3.2 Long-Term Expression

The native HSV latency-associated promoter-regulatory region has proven to be the only promoter system that supports sustained gene expression from recombinant HSV-1 vectors in the nervous system [119,200,226,227]. The functional contribution of specific *cis*-acting elements to latency-associated gene expression is currently being assessed in an attempt to build an optimized promoter based on the native LAT promoter-regulatory region. We now know that latency-specific gene expression is dependent upon sequences within the 203 bp TATA-containing LAP1 and upstream regulatory region [111,114,226,228–235] (Figure 7.4). LAP1, located immediately upstream of the 5' end of the 8.3 kb minor LAT, contains a TATA box [236–238], an ICP4 binding site [239–241], a CAAT box [229], a USF1 binding site [238,242,243], two CRE elements [236–238,244,245], an Egr-1 site [246,247], putative Sp1, YY1, and POU factor binding sites, as well as an enhancer element at the transcription initiation site that is required for basal transcription *in vitro* [238]. Binding of ICP4 at the transcription start site [239–241], or Egr-1 directly downstream of the TATA box [246,247] represses transcription, presumably by hampering preinitiation complex formation. *In vitro* studies in PC12 cells suggest that the LAT promoter is activated via the Ras/Raf signal transduction pathway in response to external factors such as NGF and sodium butyrate [248,249]. We and others have previously shown that the TATA box, USF1, and CRE sites are required for LAT expression *in vivo* [121,237,238,244], and we have recently shown that the putative POU factor binding site contributes to LAT expression *in vivo* [Soares and Glorioso, unpublished data].

LAT is also expressed late in the lytic virus life cycle, primarily by a second promoter, LAP2, which augments LAP1 in regulating LAT expression during latency [114,232]. LAP2 is located immediately proximal to the LAT intron (Figure 7.4) [226,250,251], contains an initiator element which displays homology to the TATA-less adenovirus IVa2 gene initiator [252] rather than a

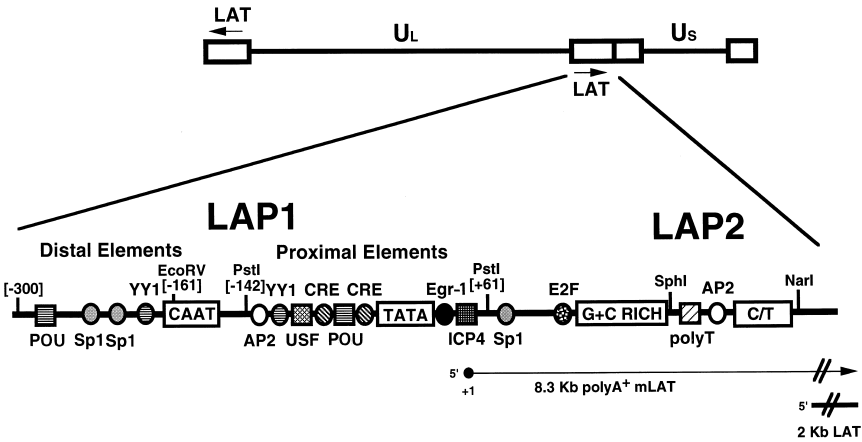


Figure 7.4. *Cis*-acting elements within LAP. Two latency active promoter (LAP) sequences, LAP1 and LAP2, have been identified upstream of LAT. LAP1 contains a TATA box with upstream control elements such as CAAT, USF1, CRE, Sp1, YY1, and POU sites, a region at the start of transcription that plays a role in LAP1 basal activity, and an ICP4 binding site that downregulates LAT expression as well as a strong enhancer element downstream of the transcription start site. LAP2 is a weaker promoter than LAP1, lacking a TATA box, but containing elements common to a wide variety of housekeeping gene promoters, a GC rich sequence and a C/T rich sequence separated by a long sequence of 23 thymidines, all of which are required for LAP2 activity in transient gene expression assays.

TATA box, and is comprised of regulatory elements found in a variety of housekeeping gene promoters, including a C/T rich element present in many TATA-less promoters [252–254], and a polyT stretch of 23 thymidines also found in putative regulatory sequences of higher eukaryotic genes [255–260]. Both the C/T element and the polyT stretch are required for LAP2 activity in transient gene expression assays [226,251]. Using EMSA and DNase footprint analyses [251], we have shown that the C/T rich element bound the transcription factor Sp1 and the protein NSEP-1 [261], which belongs to a family of factors that previously had been shown to bind the *c-myc* promoter as well as the EGF-R promoter [262–265]. Using an S1 sensitivity assay we mapped an S1-sensitive site to a region between the polyT element and the C/T rich sequence of LAP2. S1 nuclease sensitive sites often correspond to *in vivo* DNaseI sensitive sites [266,267]. DNaseI sensitive sites have been seen in 5' regions of active transcription [266, 268] and further upstream enhancer regions [269], and may function to disrupt chromatin structure by nucleosome exclusion [270–272]. We have shown that binding of HMG I(Y) protein to the polyT stretch promotes the recruitment of SP1 and perturbs the local DNA conformation [250]. The G + C rich region of LAP2 contains numerous CpG islands [273], which may alter the methylation locally. However, this

mechanism may not account for LAP activity since the viral genome does not appear to be extensively methylated during latency [274]. Together, these features of LAP2 may contribute to nucleosome exclusion or positioning effects that promote transgene expression in the context of the viral genome.

LAP1 was initially shown to provide long-term expression of β globin in mouse PNS from a vector in which the β globin cDNA was inserted immediately downstream from LAP1 [231]. However, expression was demonstrated to wane over time [275]. A similar recombinant with the α -interferon (α -IFN) cDNA inserted into the same site immediately downstream from LAP1 failed to express α -IFN during latency [209], suggesting that sequences within the first intron of β globin may have been responsible for allowing transgene expression from the β globin recombinant virus during latency. Another recombinant in which the rat β -glucuronidase cDNA was inserted downstream from LAP1 in a virus deleted for part of LAP2 expressed high levels of β -glucuronidase acutely in mouse trigeminal ganglia and brainstem yet low levels of the transgene during latency [276]. However, like the β globin recombinant, the level of transgene expression and the number of expressing neurons decreased dramatically with time. In other studies, LAP1 was unable to provide sustained transgene expression from the glycoprotein C (gC) locus [211] or even from LAT loci that lack downstream sequences including LAP2 [275]. However, fusion of the MoMLV LTR to LAP1 compensated for lack of downstream sequences permitting long-term transgene expression in the PNS [211], suggesting that LAP1 lacks elements necessary for long-term transgene expression but possesses the *cis*-acting sites required for neuronal-specific expression. Prolonged β -galactosidase expression was seen using the MoMLV LTR promoters, but not neurofilament promoter, when the *lacZ* cassette was inserted 800 bp upstream and in the opposite orientation to LAT [210], implicating the ability of this region (LAP1) to promote neuronal-specific expression when linked to long-term expression elements (MoMLV LTR or LAP2).

Insertion of the transgene immediately downstream of LAP2 in the native LAT loci [119,200] or downstream of LAP2 alone in the gC ectopic site [226] provided long-term expression in both mouse PNS (as long as 10 months) and rat CNS neurons, although the level of transgene expression in brain is reduced compared to that observed in sensory neurons of the PNS [200]. We have also shown that LAP2 is capable of long-term expression of NGF in trigeminal and dorsal root ganglia neurons (Figure 7.5) when the gene expression cassette was present in either the tk or U₃ ectopic loci [227]. These results suggest that some element(s) present within LAP2 is responsible for mediating long-term expression during latency and that the MoMLV LTR can substitute for that activity. Although LAP1 and LAP2 can function in driving transgene expression in the CNS, further modification will be required to achieve physiologic levels of therapeutic gene expression in brain.

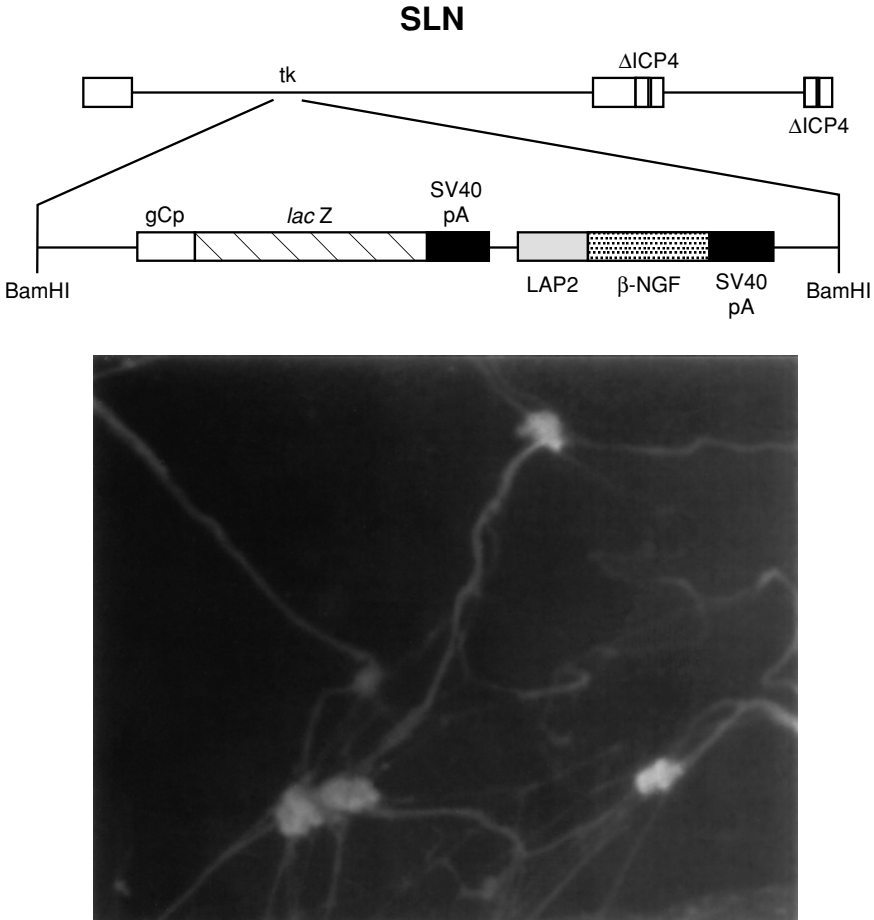


Figure 7.5. Immunofluorescent detection of HSV-1 replication-defective genomic vector-mediated β -NGF immunoreactive protein in primary DRG cultures during *in vitro* latency. Primary DRG dissociated neuronal cultures isolated from E16 rat embryos were infected (MOI=10) with SLN (ICP4⁻, tk::LAP2-NGF) and at 14 days post-infection, cell monolayers were fixed with methanol and examined for β -NGF expression by immunofluorescence using a polyclonal Ab specific for the protein and FITC-labeled secondary antibody. The LAP2 promoter was effective in driving NGF expression during *in vitro* latency at 14 days post-infection.

In addition to LAT promoter strength, the level and duration of transgene expression may also depend on mRNA translation and/or stability. In order to increase transgene expression from LAP, an internal ribosome entry site (IRES) from encephalomyocarditis virus was fused to a reporter gene construct that was introduced downstream from LAP. These recombinants

yielded long-term expression of β -galactosidase in murine sensory and motor neurons [277], although the level and site of expression varied within the population of latently infected cells. The increased levels of foreign gene expression may be due to the IRES promoting efficient transport of the message to the cytoplasm and increased translation of the message. This modification may prove particularly useful for CNS applications since the level of stable transgene expression in brain from LAP has been only detectably by RT-PCR [200].

7.3.3.3 Regulated Transgene Expression

Since vectors employing LAP have failed to achieve high levels of transgene expression in brain, we employed both constitutive and inducible recombinant chimeric transactivating systems to attain regulatable long-term gene expression in the CNS. Our first attempt at engineering an HSV vector capable of regulating transgene expression used the Gal4–VP16 potent chimeric transactivator consisting of the yeast Gal4 DNA binding domain fused to the transactivating domain of the HSV VP16 gene product. This strategy was based on the ability of HSV vector-encoded chimeric Gal4–VP16 to bind to and activate the transgene promoter containing five tandem copies of the 17bp Gal4 DNA recognition element. Gal4–VP16 has been shown to transactivate promoters containing this site [278–280] despite the repressive presence of nucleosomes [281,282]. In addition, expression of Gal4–VP16 was controlled by a Gal4-sensitive promoter creating a constitutive autoregulatory loop. This system yielded enhanced transgene expression both in culture and in brain. However, the potential autoregulatory loop did not produce constitutive long-term expression of both the transactivator and the transgene in the CNS since the promoters were only transiently active [283].

In order to target and regulate transgene expression, we have since modified this system to achieve inducible expression of the transgene. In this approach the constitutive transactivator is replaced by a chimeric molecule consisting of a mutant form of the hormone binding domain of the progesterone receptor fused to the constitutive transactivator. This mutated receptor binding domain fails to effectively bind progesterone but can efficiently bind the progesterone analog RU486 [284,285]. In the presence of RU486, the inactive chimeric transactivator assumes a conformation that enables it to bind to and transactivate a Gal4-sensitive promoter, which in this inducible system drives expression of the transgene. Thus, in the absence of RU486 the transgene is silent, yet expression can be activated by addition of the drug. Using this inducible system we have been able to achieve high levels of viral vector-derived transgene expression in rat brain upon intravenous administration of the inducing agent RU486, demonstrating the feasibility of the

drug-inducible viral vector delivery system [286]. This system can be employed to specifically activate the expression of single genes, or more importantly multiple genes to coordinate expression in the background of a totally defective genomic vector. Experiments are in progress to test the inducible system when the transactivator is expressed by LAP, where even low levels of expression may provide sufficient amounts of transactivator to produce therapeutic levels of a transgene product whose promoter is targeted by the recombinant transactivator.

7.3.4 HSV AMPLICONS

Often referred to as 'defective' HSV-1 vectors, amplicons are plasmids engineered to contain both an HSV origin of replication and packaging signals as well as a bacterial origin of replication [185]. Amplicons are propagated in bacteria and then co-transfected with a defective HSV 'helper' virus mutant to create a mixed population of HSV particles containing either the defective HSV helper genome or concatemers of the plasmid packaged within an HSV capsid (Figure 7.3B). Amplicons have been used to express reporter genes [18,181,184–186,194,202,206,219,287–289] or biologically active peptides [21,23,180,182,183,187,189–192,195,204,205,207,290–292] transiently in tissue culture systems, but the presence of helper virus particles can result in cytotoxicity, cell death, and the subsequent loss of expression.

In vivo, prolonged expression of both a *lacZ* reporter gene [20,188,193,202,206,219–221,293] and therapeutic gene products such as tyrosine hydroxylase (TH) [20], Bcl-2 [22–24], the glucose transporter [19,21,25], or HSV-TK [205] following amplicon injection into brain have been reported. In the experiments in which TH was expressed from the amplicon vector in the striatum of 6-hydroxydopamine (6-OHDA) lesioned rats [20], lesioned animals injected with the TH vector showed altered cycling following apomorphine administration. However, neither the transgene nor the vector DNA could be detected when behavioral changes in this animal model of Parkinson's disease were monitored. Since the production of amplicons require repeated passaging of the amplicon/helper virus preparation, which results in the emergence of recombinant wild-type virus estimated to occur at the frequency of 10^{-5} [20,293], these amplicon/helper preparations have resulted in the death of 10% of infected animals in experiments *in vivo* [20] and may contribute to the toxicity observed *in vitro* and *in vivo* with standard amplicon preparations. Contamination by replicating virus recombinants complicates the interpretation of these experiments particularly since such rescuers can spread to uninjected brain regions with local neuronal cell destruction. The Geller laboratory has reported the production of true helper virus-free amplicon preparations (Figure 7.3C) using multiple cosmids containing overlapping restriction fragments of the helper

virus genome which lack packaging signals [196]. However, the maximum yield obtained with that method (10^5 pfu/ml (plaque-forming units per milliliter)) was generally far lower than the titers of other vectors, and expression at 1 month was confined to an average of 35 cells/brain following intracranial inoculation [188,196]. In these recent reports expression of *lacZ* from standard amplicon preparations was reported to be negative by 30 days [188,196], in contrast to previous reports. Helper-free amplicon persisted in rat striatum while standard amplicon preparations failed to be maintained [196]. It is likely that this amplicon particle yield can be improved, which should enhance transgene expression. However, the stability and persistence of the amplicon plasmid DNA in the neuronal nucleus and gene expression over time remain to be documented.

7.3.5 NOVEL HYBRID HSV-AAV VECTORS

Novel hybrid HSV-AAV vectors have been engineered (Figure 7.3D) in order to obtain sustained gene expression in both dividing and non-dividing cells, taking advantage of the increased carrying capacity and efficient transduction rate of HSV vectors and the ability of AAV vectors to integrate into the host genome coupled with prolonged gene expression in dividing cells. Infection of dividing human U87 glioma cells in culture with HSV helper-free amplicon-AAV hybrid vectors resulted in increased duration of transgene expression compared to that observed with the helper-free amplicon system [294]. The presence of the AAV *rep* gene products increased the number of transduced cells as well as vector genome persistence, and prolonged transgene expression in the absence of *rep*-mediated toxicity. Whether this increase is due to amplification or integration of the vector genome remains to be determined, as does the utility of this hybrid system *in vivo*.

7.4 SUMMARY AND FUTURE DIRECTIONS

Herpes simplex virus possesses many features which support its utility as a gene transfer vehicle for the nervous system and its potential use in therapeutic applications for treatment of neurodegenerative disease, CNS tumors, and peripheral neuropathies. The virus naturally establishes long-term benign persistence in neurons of the brain and peripheral nervous system where the viral neuronal specific latency promoter system functions in long-term expression of the viral latency gene in the absence of host immune recognition of the infected cell. Our work has focused on further developing HSV vectors by modifying the virus to reduce vector-associated toxicity and developing a greater understanding of the factors regulating gene expression from the latent viral genome to achieve either constitutive, long-term trans-

gene expression in neurons, or regulatable, transient expression, depending on the therapeutic regimen. The current vectors are now directly applicable to treat peripheral nervous system disorders since we have shown that vector genomes persist in PNS neurons and we can achieve short-term expression from strong constitutive promoters or measurable long-term expression from the viral latency promoter system.

While the virus readily establishes latency in brain, the latency promoter system is much less active and will certainly require further modification to improve the level of promoter activity. It may be possible to increase LAP activity through the introduction of *cis*-acting sequences which are responsive to brain-derived transcription factors associated with different neuronal cell types. Additionally, the latency promoter might be employed to express an artificial transactivator capable of upregulating LAP expression through dosing of individuals with drugs capable of passing the blood-brain barrier and activating expression of the therapeutic gene product.

Applications involving conditional replication-competent vectors for cancer therapy will require considerably more research to define a suitable vector background. It may prove efficacious to target the virus to particular cell types by substituting the specific regions of the HSV envelope glycoproteins involved in binding to the natural cell-surface receptors with novel binding ligands that recognize a specific cell type *in vivo*. Thus, it may prove feasible to target the virus specifically to CNS tumor cells while eliminating binding of the vector to other neighboring neurons. Further specificity may be introduced by exploiting tumor cell-specific promoters, which may now be active in the backbone of a multiple IE gene deleted genomic vector, which would add an additional level of safety to the design of suicide vectors to attack gliomas since expression of the pro-drug activator (HSV TK or CD) would only occur in tumor cells. Although considerable progress has been achieved in designing HSV vectors for the treatment of nervous system disorders, and many vectors are now being tested in animal models of human disease, further work will be necessary for many CNS applications as well as applications employing the virus to treat disease of other tissues.

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8 Mammalian Artificial Chromosomes: Prospects for Gene Therapy

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8.1 INTRODUCTION

The efficacy of gene therapy as a means of treating disease will depend greatly upon the design of appropriate *in vivo* carriers of the introduced genes. In the cell, the element nature has designed for this purpose is the chromosome. It therefore seems a logical approach to try to imitate nature's *modus operandi*, and create a mammalian artificial chromosome (MAC).

Our understanding of the genetic components that constitute a fully functional chromosome in mammals is currently limited. Nevertheless, as discussed below, progress has been made in identifying some of these components, and strategies are being developed to generate minichromosomes, which might provide the basis for a MAC vector.

8.2 WHY BUILD A MAC?

There are numerous reasons for wanting to build a MAC. The first is that it would provide an experimental system for exploring the organisation and mechanics of mammalian chromosomes in mitotic cells. Moreover, the introduction of MACs into transgenic mice will allow the DNA requirements for mammalian chromosome function and stability during meiosis to be dissected. This work will be crucial to furthering our understanding of the requirements of correct chromosome pairing and recombination. Aside from studies on chromosome behaviour, MACs could be used for the functional analysis of genes, gene clusters and genomic regions too large to handle easily in the vector systems currently available. For example, they could be used to analyse the requirement for distant regulatory elements in gene

expression, and to analyse the sequence requirements for X chromosome inactivation and for genomic imprinting. The advantage of the MAC approach over the use of yeast artificial chromosomes (YACs) will be the lack of position effects and insertional mutagenesis arising from integration of large DNA molecules into the host genome.

At a practical level MACs could potentially provide an alternative to virus-based approaches for the correction of genetic disease by somatic gene therapy. The major advantage offered by a MAC vector for gene therapy purposes would be that the upper size limit on the DNA insert is likely to be very large, probably in the tens of millions of base pairs (native human chromosomes range in size from 45 megabasepairs (Mbps) up to 250 Mbps). Normal gene regulation is often dependent on regulatory elements that are buried in large introns or reside tens, or even hundreds, of kilobases (kb) away from the body of the gene. Since in many cases these elements may not have been fully characterised the possibility of introducing the gene in its native genomic context would ensure correct and full gene expression. This is extremely important for therapies where the goal is to regenerate native gene function, since tissue-specific expression, appropriate temporal control and accurate dosage are the critical factors in gene behaviour. In addition, large-scale MACs carrying entire cluster of genes would provide the potential to correct contiguous gene-deletion syndromes, where the defect is in more than one gene. For applications where the requirement is for a large DNA region to be introduced and permanently retained by the cell in an extrachromosomal state (hence avoiding insertional mutagenesis) with minimal risk of unwanted immune reactions against foreign proteins the MAC represents the ultimate in vectors.

8.3 THE IDEAL MAC

At its simplest, a linear eukaryotic chromosome must have a centromere, origins of replication and two telomeres. These constituents, when identified in yeast, sufficed to generate a YAC (Hahnenberger *et al.*, 1989; Murray and Szostak, 1983); whether or not the mammalian counterparts would be sufficient to generate a MAC is unknown. It is conceivable that other elements will be required in the mammalian system; for example, data is now accumulating which suggests that the microtubule–chromosome interactions that control chromosome position and mobility are not limited to the kinetochore, but rather are also distributed along the chromosome arms (Afshar *et al.*, 1995; Murphy and Karpen, 1995; Vernos *et al.*, 1995). In addition, in higher eukaryotes heterochromatin in the centric region may have a crucial biological function in ensuring correct segregation (Barton and Goldstein, 1996; Dernburg *et al.*, 1996).

If we were to consider the makeup of an ideal MAC what features would be included? Native mammalian chromosomes are very large unwieldy nucleoprotein complexes carrying thousands of genes. To be practical, a MAC would necessarily have to be orders of magnitude smaller, and for *in vitro* manipulation, while a size of a few million base pairs may be manageable, a really useful MAC vector would be no more than a few hundred kilobasepairs (kbps). To this one should have the means of adding the DNA of interest which, for maximum utility, could be of any size. Thus, a MAC should be mitotically stable whether at a size of a few hundred kbps, when fitted with a large gene, such as the 2400 kbps Duchenne muscular dystrophy locus (Heus *et al.*, 1996), or when carrying a chromosomal segment tens of millions of base pairs in length.

By mitotic stability we mean that the transmission of the chromosome to daughter cells should be similar to the robustness of native chromosomes, with a propensity for rearrangements (such as translocation, deletions or inversions) to occur at a frequency no greater than that seen with native chromosomes. Small versions of the MAC should not integrate into the larger native chromosomes at any appreciable rate.

Our ideal MAC should be invisible to the immune system. It should not carry any virally-encoded antigens or foreign proteins of any kind which could stimulate the immune system. In theory a MAC constituted of native human genetic elements would not provide a basis for immune reactions apart from what might be encoded in any added insert DNA.

The last feature to be considered here is the most problematic aspect of MAC technology. It is that the MAC should be amenable to easy delivery to the target cell population, both general cell types and very specific ones. Delivery of DNA is currently a very inefficient process (see later). It is unlikely that the MAC itself will play a role in the delivery process, but that it will be passively carried by other agents, either virally- or chemically-based. A major difficulty to be overcome is how to deliver large DNA molecules intact to the cell. Means of condensing or packaging large DNA molecules into manageable, sturdy, yet reversible conformations is an important area for research. Furthermore, once the cell membrane has been breached, the efficient transport of DNA to the nucleus is another process needing investigation.

8.4 STRATEGIES FOR GENERATING A MAC

How far are we from generating an artificial mammalian chromosome? MACs do not exist as yet, but advances have been made in the characterisation of the various functional elements. Progress has come both from the manipulation of existing human chromosomes (the so-called top-down

approach), and from attempts to assemble the various components (the bottom-up approach).

8.4.1 THE TOP-DOWN APPROACH

The use of radiation-induced breakage to generate small minichromosomes has been used to study the DNA requirements for centromere function in both fission yeast and in *Drosophila* (Murphy and Karpen, 1995; Niwa *et al.*, 1986; Sun *et al.*, 1997). In mammalian cells chromosome breakage has been induced and stable truncated chromosomes recovered in a semi-orderly fashion through the introduction of short stretches of the vertebrate telomere repeat sequence (TTAGGG) $_n$ (Barnett *et al.*, 1993; Farr *et al.*, 1991; Hanish *et al.*, 1994). Telomeric DNA can therefore be used as a tool for dissecting mammalian chromosomes, and work in this area has focused on the isolation of human centromeric DNA on replicating minichromosomes. The advantage of the approach is that it does not first require a complete understanding of mammalian chromosome structure and avoids uncertainty over whether mammalian chromosomal elements will function when introduced into mammalian cells as naked DNA or as yeast chromatin. The major disadvantage is that chromosome manipulation in a mammalian cell background is a highly inefficient process reliant either on the isolation of very rare homologous recombination events, or on the identification of fortuitous random integration events. A further limitation is that events that generate structures unstable during mitosis may not be recovered.

In general the strategy has been to manipulate a non-essential chromosome retained in a somatic cell hybrid through the use of cloned telomeric DNA, with powerful biochemical selections being applied to isolate events involving the chromosome of interest. Applying telomere-associated chromosome fragmentation to the human X chromosome, Farr and co-workers (Farr *et al.*, 1992) described the generation of a Chinese hamster fibroblast cell line carrying a truncated version of a human X chromosome from which the whole of the long arm had been removed. The chromosome was stabilised by the integration of a 'telomere-construct' into the alpha satellite array and the seeding of a new telomere at this position. This stabilising integration resulted in the loss of 750 kbps of alpha satellite DNA, leaving an array of ~2.5 Mbps with apparently full mitotic function. This is consistent with the description of naturally-arising, mitotically stable variants that have undergone large deletions of alpha satellite DNA (Wevrick *et al.*, 1990). No down-regulation of gene expression through position effects exerted by the juxtaposed centromeric heterochromatin was observed (Bayne *et al.*, 1994). This suggests that the physical proximity of exogenous selectable markers (driven by strong viral promoters) and functional chromosomal elements need not be a limiting feature in this system. Whether the same applies to other genes and

promoters remains to be seen. More recently, the same chromosome has been subjected to a further round of fragmentation, resulting in a small linear human chromosome that is < 10Mbps in size in which the centromere is flanked by two introduced telomere constructs. This minichromosome appears to be as stable mitotically as the whole X chromosome from which it was derived (Farr *et al.*, 1995). A modified version of this strategy has been used to produce both long and short arm truncations of a human Y by targeting telomeric DNA to the alpha satellite DNA at the Y centromere (Brown *et al.*, 1994).

A considerable amount of information on the sequence composition of the human Y centromere has emerged from the analysis of naturally arising rearranged Y chromosomes. This has shown that the sequences necessary for Y centromere function can be localised to an interval containing ~ 200 kbps of alpha satellite and 300 kbps of adjacent short arm sequences (Tyler-Smith *et al.*, 1993), a conclusion supported by the dissection of the Y centromere using cloned telomeres. Studies in other eukaryotes (*Schizosaccharomyces pombe* and *Drosophila melanogaster*) have also suggested the requirement for flanking DNA sequences on minichromosomes for full mitotic segregation (Baum *et al.*, 1994; Murphy and Karpen, 1995). More recently the Oxford group have manipulated their Y chromosome derivatives with further rounds of fragmentation to generate Y-derived linear minichromosomes ranging in size from about 9 Mbps down to 3 Mbps. The smaller minichromosomes are all derived from Yq and their alpha satellite DNA has undergone extensive rearrangement. A minichromosome of 4 Mbps appears to be mitotically stable over several months in CHO cells, while a smaller 2.5 Mbps derivative showed signs of instability (Brown *et al.*, 1996; Heller *et al.*, 1996). When introduced into mouse embryonic stem cells the various human minichromosomes transferred (ranging from 4 to 15 Mbps in size) were found to rearrange or segregate aberrantly and to be rapidly lost in the absence of selection (Shen *et al.*, 1997; Loupart *et al.*, 1998). The reason for this poor segregation is as yet unclear, but one possibility is that established cell lines may have less stringent controls over centromere function and chromosome segregation than normal embryonic cells. Obviously the mitotic stability of any minichromosome in normal primary cells, as opposed to established tissue culture cell lines, is a critical aspect. At present the approaches available to mammalian geneticists for studying the behaviour of chromosomes in living cells are limited in their usefulness, and more robust assay systems are needed.

The minichromosomes described to date can be shuttled between different cell lines using microcell-mediated chromosome transfer (McNeill Killary and Lott, 1996) and are approaching a size where transfer into yeast (where the efficiency of homologous recombination would considerably simplify further manipulations) might be considered. However, whether

such structures with their extensive arrays of tandem repeats would be stable in *Saccharomyces cerevisiae* and whether yeast chromatin will function when transferred back into a mammalian system remain uncertain. One possible way around some of the limiting aspects of manipulating chromosomes in established mammalian cell lines has recently been presented; this is the use of the avian leukosis virus- (ALV-) induced B cell lymphoma cell line DT40 (Brown *et al.*, 1996). DT40 is unusual for a vertebrate cell in that it displays very high levels of homologous recombination, even when non-isogenic DNA is used and without selective enrichment (Buerstedde and Takeda, 1991). In DT40 targeting efficiencies of 10–100% of transfectants have been routinely reported, orders of magnitude higher than those observed in mammalian cells. By transferring mammalian chromosomes into the DT40 background systematic manipulation of the target is possible (Dieken and Fournier, 1996; Dieken *et al.*, 1996). Targeted chromosome fragmentation has already yielded a ~2.5Mbps human X cen-based minichromosome which is stable in DT40 cells when maintained for several months off selection (Mills *et al.*, 1999). Minichromosomes can then be shuttled back into mammalian cell lines in order to assess the effect of the various modifications on mitotic stability. Using this strategy it may be possible to modify candidate minichromosomes further in the DT40 environment, avoiding the need to assemble a MAC from cloned constituents.

Along conceptually similar lines some groups have identified naturally-arising dot chromosomes, which can be used as the basis for further manipulations. Although several such marker chromosomes have been described in the literature, their analysis has often not been taken beyond the cytogenetic level, with no accurate information about their mitotic stability, or their size and structure available. For example, a somatic cell hybrid retaining a small stable derivative of human chromosome 1 was described more than a decade ago. Based on slot blots probed with repetitive DNA the human content of the hybrid cell line was estimated to be < 0.05%. This suggested that the minichromosome retained ~1000–2000 kbps of chromosome-specific DNA together with a reduced centromeric domain (Carine *et al.*, 1989). More recently, an Italian group has described a marker derived from human chromosome 9. This chromosome has been transferred by MMCT into a rodent cell line and tagged by targeting a selectable neomycin resistance gene into the alpha satellite DNA. This chromosome has been estimated to be in the 5–10 Mbps size range (Raimondi *et al.*, 1995). The main difficulties with this approach are, firstly, the need to define the origin of the DNA sequences present on the marker, which may have undergone considerable rearrangement during its genesis, and, secondly, the need to determine the size and nature of the chromosome (i.e. whether it is linear in structure or exists as a ring).

8.4.2 THE BOTTOM-UP APPROACH

A bottom-up approach was used to construct YACs since the minimal elements required for proper function were known and had been isolated (Hahnenberger *et al.*, 1989; Murray and Szostak, 1983). In mammals, however, only the telomere has so far been defined at the DNA level. One can place short arrays (a few hundred base pairs) of the vertebrate telomere repeat [TTAGGG] n on the vector and these will seed the formation of new chromosome ends in established cell lines (Barnett *et al.*, 1993; Farr *et al.*, 1991; Hanish *et al.*, 1994; Taylor *et al.*, 1994). However, whether this will be sufficient to provide telomere function in primary cells is as yet untested.

Although it seems that origins of replication occur relatively frequently along the chromosome (an origin is estimated to be present every 50–300 kbps) the precise DNA sequence requirements (if these exist) of mammalian origins, or how the point at which DNA replication initiates is influenced by neighbouring chromosomal loci, have not been clearly defined. Since it seems likely that any segment of mammalian DNA > 100 kbps will be replication-competent in a chromosomal context the problem of defining a mammalian origin of replication can at present be sidestepped (Calos, 1996; Coverley and Laskey, 1994; Tyler-Smith and Willard, 1993).

A more pressing obstacle is our lack of understanding of the makeup of a minimal mammalian centromere. The most likely candidate for a DNA sequence which plays a functional role at the human centromere is the alpha satellite DNA, found at the primary constriction of all normal human chromosomes. The amount of alpha satellite DNA present at the centromeric region varies widely (from 300 kbps on some Y chromosomes, to many Mbps on some autosomes) (Tyler-Smith and Willard, 1993; Willard, 1990). Rearrangements of alpha satellite arrays suggest that the amount required for a minimal functional centromere may in fact be relatively small; for example, a human Y-derived minichromosome has been generated experimentally which retains only 140 kbps of alpha satellite DNA (Brown *et al.*, 1994; Heller *et al.*, 1996). One approach to the centromere problem has been to transfect alpha satellite DNA into established mammalian cell lines, such as CHO cells, human HT1080 and mouse L cells (Haaf *et al.*, 1992; Larin *et al.*, 1994; Taylor *et al.*, 1996). A variety of observations were reported, including unstable extrachromosomal elements, chromosome instability, secondary constrictions, CREST antisera binding and anaphase bridges. This suggested that alphoid DNA is capable of mimicking at least some of the features of a human centromere, but failure to observe full centromere function left its role unresolved. More recently, however, two groups have described the creation of stable *de novo* minichromosomes at low frequency in tissue culture cells (Harrington *et al.*, 1997; Ikeno *et al.*, 1998). In the first report large synthetic arrays of BAC-cloned alpha satellite DNA (of the type found at either human

chromosome 17 or the Y centromere) were combined with telomeric repeat DNA and genomic carrier and lipofected (without a pre-ligation step) into human HT1080 cells. This led to the generation of a small number of linear 6–10 Mbps microchromosomes, which segregated well and were maintained at single copy. Subsequent detailed cytogenetic analysis revealed that in most cases these microchromosomes were composed of DNA originating from the recipient genome in addition to the transfected sequences (often through telomere-mediated truncation events). In one case, however, the microchromosome did not contain any detectable alphoid DNA other than that introduced in the transfection mix, suggesting that it might have been created *de novo*. In the second report YACs carrying alpha satellite DNA from human chromosome 21 were retrofitted with vertebrate telomere repeat and a blasticidin resistance selectable marker. The YAC DNA was purified by pulsed field gel electrophoresis and either lipofected or microinjected into HT1080 cells. In some transfectants minichromosomes were observed. These minichromosomes were estimated to be several Mbps in size, apparently consisting of multimers of the ingoing YAC, but the structure – linear or circular – has not been ascertained. More recently this YAC approach to generating human artificial chromosomes has been repeated by others (Henning *et al.*, 1999). Together these experiments strongly suggest that alpha satellite DNA may be sufficient to provide mitotic centromere function in human cells. However, the low frequency of the events and the difficulty in fully defining the composition of the resultant minichromosomes means that the role of alpha satellite DNA and the requirements for an artificial minichromosome remain to be fully elucidated.

An alternative approach to the identification of a minimal mammalian centromere has been pioneered by Vos and co-workers. They have used the ability of the Epstein–Barr virus (EBV) encoded EBNA-1 protein to interact with the its cognate *oriP* sequence to retain linked DNA in a mammalian nucleus. After constructing large circular fragments linked to *oriP* (100–300 kbps in size), the stable maintenance of these episomes in cells which are also expressing EBNA-1 was observed (Sun *et al.*, 1994). More recently circularised human insert-containing YACs have been retrofitted with *oriP* sequences, allowing their maintenance in appropriate cell lines (Simpson *et al.*, 1996). In theory, it should be possible to construct a library of large genomic fragments, perhaps enriched for alpha satellite DNA, which could be tested for the presence of centromeric function following the removal of EBNA-1 by, for example, the use of a site-specific recombinase (Kilby *et al.*, 1993).

Intriguingly, several human marker chromosomes have been reported which are relatively stable mitotically while lacking alpha satellite DNA. It appears that occasionally rare epigenetic events can spontaneously activate regions on chromosome arms to provide centromere/kinetochore function

without any change at the level of the DNA sequence (Choo, 1997; du Sart *et al.*, 1997). Evidence for epigenetic influence on centromere stability has also emerged from studies in the yeast *S. pombe* (Steiner and Clarke, 1994) and is *Drosophila* (Williams *et al.*, 1998). Indeed, it seems increasingly likely that critical aspects of the centromere/kinetochore structure may reside in unique features of the centromeric chromatin and may be partially independent of the underlying primary DNA sequence.

8.5 PRACTICAL PROBLEMS AND UNKNOWNNS

A vital consideration of any approach for generating MACs is whether a stable chromosome can be generated that consists only of a functional centromere domain and replication origins flanked by newly seeded telomeres. The spatial concentration of the various chromosomal elements into such a small structure may result in functional interference. Moreover, the extent to which extracentric DNA on the chromosome arms is necessary for normal spindle interactions and correct chromosome segregation is currently unknown. As mentioned above, recent evidence suggests that motor proteins are positioned along the length of the chromosome arms and may be involved in the stabilisation of the bipolar spindle and the proper positioning and segregation of chromosomes. If the presence of chromosome arms is crucial for stability then this may define the minimal size of a functional linear mammalian chromosome. In the early studies on YACs it was discovered that chromosome stability and segregation were strongly dependent on chromosome size, with YACs of less than 100 kbps being highly unstable (in *S. cerevisiae* the natural chromosomes range in size from 245 kbps up to 2.2 Mbps) (Murray *et al.*, 1986). Together the top-down and bottom-up approaches should allow the minimal size requirements for a linear mammalian chromosome to be mitotically stable to be ascertained (Brown *et al.*, 1996; Heller *et al.*, 1996; Masumoto *et al.*, 1998; Mills *et al.*, 1999).

Once a basic MAC vector has been produced two major challenges exist before its use in somatic gene therapy can be seriously contemplated: firstly, the manufacture of the MAC in quantities sufficient for gene therapy purposes, and secondly, delivery to the cell. Large DNA fragments are difficult to handle and deliver in an intact state. Whole cell fusion using polyethylene glycol has been used to introduce large YAC molecules intact into tissue culture cells, but appears to work only for some rodent cell lines and would not be suitable for delivery of MACs *in vivo* (Huxley *et al.*, 1991; Larin *et al.*, 1994). Microcell-mediated chromosome transfer can be used to shuttle mammalian chromosomes between a variety of cell types, but it is a highly inefficient process and again could only be applied *in vitro*. Viral packaging of DNA is likely to place an upper limit of a few hundred kbps on the DNA

molecule that can be delivered. Some other methods exist for which the upper size limit has not yet been established, such as delivery by particle bombardment (Cheng *et al.*, 1993), direct microinjection of DNA into the cell (Gnirke *et al.*, 1993), cationic lipids and receptor-mediated uptake (Cristiano *et al.*, 1993; Curiel, 1994; Gao *et al.*, 1993). A cationic lipid such as lipofectin has been used to deliver at least several hundred kbps of DNA intact to cells in tissue culture; it is relatively non-cytotoxic and seems at present to be the most likely delivery system applicable *in vitro* and *in vivo* (Caplan *et al.*, 1995; Strauss *et al.*, 1993; Stribling *et al.*, 1992; Zhu *et al.*, 1993). Another complicating aspect is that at present it is unknown whether the MAC will have to be delivered as chromatin or whether it can be isolated as naked DNA. A further challenge will be the production of sufficient quantities of the MAC. Although some selectable markers exist which may allow for the selection of multiple copies per cell through the application of specific drugs (e.g. the dhfr locus and methotrexate selection) the level of amplification is likely to be quite modest. It is difficult, therefore, to envisage how, at present, the problems of limited material together with the highly inefficient delivery methods will be overcome once MACs are available. There is no doubt that major technological and conceptual breakthroughs will be required before the idea of using MACs in somatic gene therapy can be turned into a practical reality.

8.6 CONCLUDING REMARKS

A mammalian artificial chromosome will consist of the various functional elements, either cloned or synthesised versions, and should retain no genes, apart from essential marker genes introduced as part of the vector. It will be fascinating to see whether this structure arises out of the 'cut-and-paste' approach, as used for the development of yeast artificial chromosomes, or from extensive manipulation and modification of minichromosomes generated in a vertebrate cell system. Both approaches will add to our understanding of mammalian chromosome structure and its relationship to function.

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9 Gene Therapy for Severe Combined Immunodeficiency

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9.1 INTRODUCTION

Severe combined immunodeficiencies (SCIDs) are a group of inherited disorders characterised by a profound reduction or absence of T lymphocyte function (reviewed in Fischer, 1991). The resulting deficits in both cell-mediated and humoral immune responses invariably lead to premature mortality in the absence of haematopoietic stem cell transplantation. Since the first successful human lymphocyte antigen (HLA) matched bone marrow transplants (BMT) were performed in two primary immunodeficiency disorders over thirty years ago, transplantation technology has advanced considerably, and the cure rate for SCID using matched sibling donors is now over 95%. However, for only 30% of patients does such a donor exist, and for T cell depleted haploidentical parental grafts, the success rate falls to 50% (Fischer *et al.*, 1990). Complications primarily relate to toxicity arising from the conditioning regimen, pre-existing infection and delayed reconstitution of immune function post-transplant. Recently, *in utero* transplantation of haploidentical cells has attempted to pre-empt the development of clinical disease, and to utilise the proliferative and possibly tolerogenic foetal environment to facilitate engraftment (Flake *et al.*, 1996; Wengler *et al.*, 1996). The efficacy and safety of this approach awaits comparison with conventional transplantation, and remains limited to those families with previously affected children.

9.2 MOLECULAR PATHOLOGY OF SCID

SCID comprises a genetically heterogeneous group of conditions, the molecular lesions of which are increasingly becoming defined (Table 9.1).

Table 9.1 Genetics of SCID

Disease	Cells affected	Defective gene and/or protein	Chromosomal location
X-linked SCID	T, B(?), NK	γ c	Xq13.1
Autosomal recessive SCID	T, B, NK(?)	ADA	20q12–13.11
	T, B, NK(?)	PNP	14q11.2
	T(?)	CD3 ϵ /CD3 γ	11q23
	T	ZAP 70	2q12
	T, NK	JAK-3	?
MHC class II deficiency	T, B	RAG1/RAG2	11p13
	T, B	RFX5	?
	T, B	RFXAP	?
	T, B	CIITA	?
MHC class I deficiency	T, B	TAP2	6p21.3

9.2.1 X-LINKED SEVERE COMBINED IMMUNODEFICIENCY

The X-linked form of SCID (X-SCID) is often distinguishable from other forms by its characteristic pattern of inheritance and the observation that affected boys often have apparently normal levels of B cells (T – B + SCID), although these are intrinsically abnormal. X-SCID accounts for about 50–60% of all cases of SCID and is caused by defects in the interleukin-2 receptor (IL-2R) γ chain gene (reviewed in Leonard, 1994). Originally identified as a component of the high and intermediate affinity IL-2R that is required to achieve full ligand binding affinity and internalisation, the γ c (common gamma) chain is expressed constitutively in haematolymphoid cells and is now known to be a component of additional cytokine receptors (IL-4R, IL-7R, IL-9R and IL-15R) (reviewed in Leonard, 1994). The γ c chain is a transmembrane glycoprotein with a number of structural motifs characteristic of cytokine receptor superfamily members. It is postulated that in each of these receptors the unique chain recognises the specific cytokine, and thus confers the signalling specificity, while the γ c chain transduces the signal via its cytoplasmic domain. The different receptors are involved at various stages of T cell (including natural killer (NK) cell) and B cell growth and maturation, and the combined effect of disrupting their expression probably augments the immune defect arising from isolated IL-2R dysfunction.

9.2.2 JAK-3 DEFECTS

The JAK-3 kinase is known to associate with γ c chain-containing cytokine receptors, which makes it a candidate gene for autosomal recessive T – B + SCID. JAK-3 mutations have now been identified in a small number of T – B +

SCID patients with equivalent immunophenotypes to X-SCID, including blocked NK cell differentiation (Macchi *et al.*, 1995; Russell *et al.*, 1995).

9.2.3 ADENOSINE DEAMINASE AND PURINE NUCLEOSIDE PHOSPHORYLASE DEFICIENCIES

Many of the autosomal recessive forms of T – B – SCID are caused by defects in adenosine deaminase (ADA) (15–25% of all cases of SCID) and purine nucleoside phosphorylase (PNP) (4% of all cases of SCID), enzymes involved in purine metabolism.

9.2.4 RECOMBINATION ACTIVATING GENES (*RAG1* AND *RAG2*) DEFECTS

As many as half of T – B – SCID patients may be affected by mutations in the *RAG1* and/or *RAG2* genes (Schwarz *et al.*, 1996). These proteins are involved in the process of VDJ rearrangement, which is essential for production of functional immunoglobulin and T cell receptor (TCR) proteins. The phenotype of these patients is very variable, with absence of B cells and absent or low numbers of T cells. When T cells are present they are either as a result of maternal–foetal engraftment or a less severe mutation giving a ‘leaky’ phenotype.

9.2.5 ZAP 70 DEFECTS

Mutations in ZAP 70, a TCR-associated protein tyrosine kinase that is associated with the ζ chains of the TCR–CD3 complex, produce a distinctive form of SCID (reviewed in Perlmutter, 1994). This rare autosomal recessive disease is characterised by absence of CD8+ and abundance of CD4+ peripheral T cells (CD4+CD8 – B+ SCID), which appears to be due to an intrathymic block predominantly in the differentiation of CD8+ cells. The CD4+ T cells, however, fail to proliferate following TCR stimulation. It is thought that absence of ZAP 70 protein function results in an inability to couple the TCR to more downstream signalling events.

9.2.6 MHC CLASS I DEFICIENCY (TYPE 1 BARE LYMPHOCYTE SYNDROME)

Two siblings of consanguineous parents have been described with a reduced level of major histocompatibility complex (MHC) class I expression at the cell surface, although the molecules were present intracellularly. The genetic defect resulted from a mutation in the *TAP2* gene, a component of the TAP peptide transporter complex associated with antigen processing (de La Salle *et al.*, 1994).

9.2.7 MHC CLASS II DEFICIENCY (TYPE 2 BARE LYMPHOCYTE SYNDROME)

MHC class II deficiency is an autosomal recessive form of SCID characterised by the presence of normal levels of dysfunctional T and B cells (T + B + SCID). All bone marrow-derived cells in affected individuals fail to express MHC class II antigens (DR, DP and DQ).

This lack of expression is the consequence of a failure to synthesise the α and β chain proteins. The defect is not in the class II genes themselves and does not appear to be located within the MHC locus but appears to be in the regulation of expression of the class II genes. There is a specific defect in the binding of a protein complex, RFX, to the highly conserved X box of the MHC class II promoter in some of these patients (reviewed in Durand *et al.*, 1997). Mutations have now been found in one of the subunits of this complex, RFX5, in one group of patients and in a second component, RFXAP, in another group (Durand *et al.*, 1997 and references therein). In a third group of patients who do not display this defect, mutations have been found in another protein, CIITA, which acts as an MHC transactivator (reviewed in Durand *et al.*, 1997). This novel regulatory gene is essential for the constitutive expression of all MHC class II genes in B lymphocytes.

9.2.8 TCR-CD3 ABNORMALITIES

Rarely, defective expression and function of the TCR-CD3 complex of T cells can occur as a result of mutations in the γ or ϵ genes of the CD3 complex. The phenotype of this disease varies from full-blown SCID to mild respiratory symptoms or no apparent effects.

9.3 SOMATIC GENE THERAPY FOR SCID

Although rare, primary immunodeficiencies are in many ways ideally suited to the application of this technology, and have become model conditions on which to design and test gene therapy protocols. Rapid progress in understanding the molecular basis for some of these disorders, including adenosine deaminase deficiency (ADA-SCID), γ c chain deficiency (X-SCID), JAK-3 kinase deficiency, and ZAP 70 deficiency, has facilitated the development of therapeutic strategies based on genetic manipulation of autologous cells (Blaese *et al.*, 1995; Bordignon *et al.*, 1995; Candotti *et al.*, 1996; Kohn *et al.*, 1995; Taylor *et al.*, 1996). ADA-SCID in particular has been subject to the most preclinical investigation, and has recently led to the initiation of several clinical trials.

9.3.1 CLINICAL GENE THERAPY FOR ADA-SCID

ADA-SCID is secondary to genetic defects in the purine catabolic enzyme ADA (reviewed in Hirschhorn, 1990). In humans this primarily results in immunological dysfunction (T and B lymphopaenia), although ADA-deficient mice generated by gene targeting die perinatally from severe liver cell degeneration, indicating a critical role for ADA during murine foetal development (Migchielsen *et al.*, 1995; Wakamiya *et al.*, 1995). Interestingly, liver dysfunction reversible with enzyme replacement therapy has recently been reported in ADA-SCID (Bollinger *et al.*, 1996). Of all tissues, the highest enzymatic activity of ADA is found in immature thymocytes, and deficiency results in impaired intrathymic differentiation. Accumulation of deoxyadenosine is accompanied by preferential conversion to the toxic compound deoxyadenosine triphosphate (dATP), particularly in T cells. Biochemical studies suggest that accumulation of dATP leads to inhibition of ribonucleotide reductase (Cohen *et al.*, 1983), a key enzyme in the synthesis of deoxynucleotides, and irreversible inactivation of S-adenosyl-methionine hydrolase (SAH), which mediates important methylation processes (Hershfield and Kredich, 1979). Despite ubiquitous constitutive expression of ADA, lymphocytes are particularly susceptible to toxicity from accumulating metabolites, possibly because of predominant inhibition of T cell-specific molecules during intrathymic differentiation (Benveniste *et al.*, 1995).

Enzyme replacement therapy with bovine ADA conjugated to polyethylene glycol (PEG-ADA) has resulted in significant improvement in lymphocyte cell numbers and function in many patients, but the response is variable, and may be transient (Hershfield, 1995; Hershfield *et al.*, 1993). This probably reflects the main site of PEG-ADA activity, which is extracellular and which only partially complements the cellular deficiency. Complete absence of ADA results in lethality before two years of age. However, retention of only 1–5% of activity often leads to attenuated disease, and individuals with as little as 10% activity have been found to be immunologically normal (Hart *et al.*, 1986; Hirschhorn, 1990; Morgan *et al.*, 1987). Levels of ADA fifty times above normal have also been associated with normal immunity (Valentine *et al.*, 1977). These considerations have important implications for the design of gene therapy vectors, but suggest that relatively simple expression systems may be efficacious for this condition. Furthermore, spontaneous reversion to normal *in vivo* of inherited mutations (resulting in somatic mosaicism) has recently been demonstrated in two patients with attenuated disease (ADA-SCID and X-SCID), suggesting that corrected cells have a distinct growth and differentiation advantage over mutants (Hirschhorn *et al.*, 1996; Stephan *et al.*, 1996).

9.3.2 T LYMPHOCYTE GENE THERAPY FOR ADA-SCID

The ability of HLA-matched genotypically identical stem cell transplants for severe ADA deficiency to selectively reconstitute the lymphoid compartment in the absence of cytoablative conditioning suggested that biochemical correction of autologous T cells by somatic gene transfer could provide a similar therapeutic outcome. This was supported by preclinical studies which demonstrated a survival advantage of transduced ADA-SCID T cells in immunodeficient BNX mice (Ferrari *et al.*, 1991). The first ever approved clinical trial of gene therapy began in 1990 at the NIH, and involved repeated *ex vivo* transduction of autologous peripheral blood T cells with a retroviral vector expressing human ADA, followed by expansion and reinfusion into two patients (Blaese *et al.*, 1995). Although both patients had attenuated disease, at the time of enrolment both were lymphopaenic, anergic, and had depressed immune reactivity to specific antigens despite receiving optimal PEG-ADA enzyme replacement therapy. Within 5 to 6 months of gene therapy, the lymphocyte count in one patient had risen into the normal range, and remained stable for at least 2 years after the last infusion of transduced cells. Levels of ADA in blood lymphocytes stabilised at roughly half that found in heterozygote carriers, and was associated with return of DTH skin test reactivity, cytolytic responses, and antibody production. The second patient also responded with an initial increase in peripheral T lymphocyte numbers to levels in the high normal range, although these fell over time to levels just above those at enrolment. In contrast to the first patient, ADA levels remained very low, correlating with low gene transfer efficiency ($< 10\%$ as efficient as the other patient). Some return of immune reactivity was demonstrated, but could have arisen as a result of infusion of an expanded T cell population, and was variable anyway prior to enrolment.

This trial demonstrates the potential efficacy of gene transfer to autologous T cells for therapy of ADA-SCID, and at the present time is suggestive of clinical benefit. The outcome is confused by the concurrent administration of exogenous PEG-ADA, which to a variable extent complements the cellular immunodeficiency. Discontinuation of this treatment would seem to be the obvious test for efficacy of gene transfer, but normalisation of T lymphocyte ADA levels alone may also be insufficient to fully correct the immunodeficiency, and may necessitate continued therapy. One concern with T lymphocyte gene therapy is the potential for insertional mutagenesis arising from multiple integration events during the transduction procedure. At present there is no evidence to suggest this has occurred. Interestingly, attempts to utilise cytokine mobilised peripheral blood progenitor cells from ADA-SCID patients for gene transfer has been limited by poor yield (Sekhsaria *et al.*, 1996).

9.3.3 STEM CELL GENE THERAPY FOR ADA-SCID

The optimal target cell for curative gene therapy of many haematopoietic disorders is the pluripotent haematopoietic stem cell (PHSC). Much interest has therefore been directed towards attempts to achieve this goal for ADA-SCID. Preliminary experiments demonstrated that reconstitution of ablated murine haematopoiesis with retrovirally transduced cells could be achieved at relatively high efficiency, and that expression of human ADA persisted in secondary and tertiary transplant recipients, implying that PHSCs were successfully modified (Lim *et al.*, 1989; Osborne *et al.*, 1990; van Beusechem *et al.*, 1990; Wilson *et al.*, 1990). Several groups also demonstrated successful gene transfer to rhesus monkey bone marrow, and long-term expression of both human and murine ADA *in vivo*, albeit at low levels (< 3%) (Bodine *et al.*, 1993; Kantoff *et al.*, 1987; van Beusechem *et al.*, 1993).

The first attempt in a clinical setting to transduce bone marrow cells from a patient with ADA deficiency was conducted by Bordignon and colleagues in Milan, and was combined with reinfusion of genetically modified peripheral blood lymphocytes (Bordignon *et al.*, 1995). In this study, two retroviral vectors, distinguishable by molecular techniques, were used to transfer an ADA minigene into either bone marrow or lymphocytes from two patients with attenuated ADA-SCID who had become immunologically refractory to exogenous enzyme replacement. Administration of genetically modified cells resulted in normalisation of lymphocyte counts, antigen-specific immune responses, and mitogen- and antigen-specific proliferation. The T cell repertoire (V_{β}) was also shown to progressively normalise. Significantly, one year after discontinuation of gene therapy, peripheral T cells derived from the transduced lymphocyte population were gradually replaced by T cells derived from bone marrow, suggesting a proliferative advantage. Both children enrolled in this trial continued to receive enzyme replacement therapy, although relative dosages were decreasing. Withdrawal of PEG-ADA should reveal the true efficacy of gene transfer, and may enhance the proliferative advantage of corrected cells.

In a separate study, CD34+ cells obtained from the bone marrow of three children with ADA deficiency were used as targets for transduction by a retroviral vector encoding the human ADA cDNA (Hoogerbrugge *et al.*, 1996). Mononuclear cells and granulocytes retaining the vector genome were detectable by polymerase chain reaction (PCR) in the peripheral circulation for up to three months after gene transfer, and in one patient in the bone marrow at six months. Detection of the vector genome after this period of time was not possible. One patient did not receive enzyme replacement until three months after infusion in an attempt to maximise proliferative advantage, although it is likely that because gene transfer efficiency was very low, this was not a sufficient time period for genetically corrected clones to reveal themselves.

9.3.4 LIMITING FACTORS FOR STEM CELL GENE THERAPY

The inefficiency of retroviral transduction of the human PHSC population has been highlighted in clinical studies using marker genes. In lethally irradiated murine models, 30–40% of cells participating in long-term engraftment of the animal can be stably transduced *ex vivo* by the current generation of retroviral vectors (Karlsson, 1991; Miller *et al.*, 1994; Williams, 1990). However, transfer of this technology to humans, non-human primates or other large outbred animals has been much less successful (Bodine *et al.*, 1993; Kantoff *et al.*, 1987; Kiem *et al.*, 1994; van Beusechem *et al.*, 1993). In clinical studies, typically fewer than 1% of peripheral blood cells are marked long term by retroviral vectors (Brenner *et al.*, 1993; Dunbar *et al.*, 1995). The reasons for this discrepancy are uncertain, but probably reflect incomplete understanding of culture conditions required to maintain the integrity and functionality of the PHSC, an inability of the current generation of murine retroviral vectors to transduce quiescent or slowly dividing cells, and a deficiency of receptors on the PHSC surface for the commonly used amphotropic retroviral envelope (Orlic *et al.*, 1996).

The requirement for the current generation of retroviral vectors to transduce actively dividing cells may in particular be a limiting factor because PHSCs are relatively quiescent (Abkowitz *et al.*, 1995). Cells harvested from umbilical cord blood may be better targets for retrovirus-mediated gene transfer, and were therefore utilised in a recent clinical trial for ADA-SCID. In this study, three prenatally diagnosed infants received infusions of transduced cord blood CD34+ cells, at which time they were commenced on enzyme replacement therapy. Eighteen months after transplantation the proportion of lymphocytes retaining the transgene, and levels of ADA activity in unselected cells was extremely low (Kohn *et al.*, 1995). Gradual withdrawal of PEG-ADA may facilitate the proliferation of gene-corrected cells.

The discrepancy between results obtained from clinical trials and preclinical studies may lie partially in the requirement for ablative preconditioning of recipient animals. It is conceivable that reconstitution with genetically corrected cells would be enhanced by a controlled conditioning regimen to allow 'space' for engraftment, although the risks associated with the procedure in SCID would undoubtedly be increased.

9.4 ALTERNATIVE VECTOR SYSTEMS

Adeno-associated virus (AAV) is a non-pathogenic human parvovirus which has attracted considerable interest as a gene transfer vector (Kotin, 1994). Replication of this virus is usually dependent on co-infection with a helper

virus. In the absence of helper virus (usually adenovirus) the wild-type AAV genome can integrate stably into the host cell genome by non-homologous recombination, usually in a tandem head to tail orientation (Kotin *et al.*, 1992; Samulski *et al.*, 1991). Analysis of flanking sequences from latently infected cells has shown that integration occurs at multiple sites within a single specific locus, AAV-S1, in 60–70% of cases, which maps to human chromosome 19q13.3-qter. AAV vectors in which the *rep* gene is deleted probably integrate randomly, suggesting that *rep* gene products (Rep78 and Rep68) are important for this process.

Vectors based on AAV have been used successfully to transduce dividing cells *in vitro* and non-dividing cells *in vivo* (Ali *et al.*, 1996; Fisher *et al.*, 1997; Kaplitt *et al.*, 1994; Kessler *et al.*, 1996; Thrasher *et al.*, 1995; Xiao *et al.*, 1996). Transduction efficiency of cultured primary human fibroblasts in S-phase has been shown to be about 200 times greater than that of resting cells, although in these experiments the vector genome was shown to persist for at least 12 days in culture, and could be recruited for transduction at any time (Russell *et al.*, 1994). This property may be useful for transduction of slowly dividing cell populations such as PHSCs. However, production of recombinant AAV remains difficult, and some cell types, including CD34+ haematopoietic progenitors, may be relatively refractory to stable transduction (Goodman *et al.*, 1994; Malik *et al.*, 1997; Walsh *et al.*, 1994; Zhou *et al.*, 1994). Although the nature of the cellular receptor for AAV is unknown, the problem may lie in the ability of the cell to convert the incoming single-stranded rAAV genome to a transcriptionally active double-stranded template. This process has recently been shown to be enhanced by ORF6 of adenovirus E4 (Ferrari *et al.*, 1996; Fisher *et al.*, 1996), and by herpes viruses (Thrasher, unpublished results). Although the cellular processes that are responsible have not yet been elucidated, administration of genotoxic agents and physical stress can substitute for this helper activity, suggesting a role for host cell polymerases and repair mechanisms (Alexander *et al.*, 1994; D. Russell *et al.*, 1995). Whether the efficiency of PHSC transduction can be enhanced by similar mechanisms is at present unknown.

9.5 PRE-CLINICAL ASSESSMENT OF HAEMATOPOIETIC STEM CELL GENE TRANSFER

One difficulty in preclinical assessment of gene transfer technology is the ability to test the human PHSC population for repopulating activity. Primitive haematopoietic progenitors can be quantitated *in vitro* in long-term marrow cultures by detection of cobblestone area forming cells (CAFC) or clonogenic cells in the long-term culture initiating cell assay (LTC-IC). However, these cells represent a heterogeneous population, and may not

necessarily be representative of PHSC activity. A more informative assay, and one which tests repopulating ability, is based on transplantation of human haematopoietic progenitors into immunodeficient *bg/nu/xid* mice (Nolta *et al.*, 1994), or *scid/scid* mice (spontaneous mutation in DNA protein kinase catalytic subunit) producing chimeric SCID-human (SCID-hu) haematopoiesis (Dick *et al.*, 1991; Shultz *et al.*, 1995). These studies have shown that very primitive human cells engraft the murine microenvironment, and in the presence of human cytokines proliferate and differentiate into multiple myeloid, erythroid and B cell lineages which are maintained for at least 4 months. More recently NOD/LtSz-*scid/scid* (NOD/SCID) mice (created by backcrossing the *scid* mutation onto the NOD/LtSz diabetic strain background) have been shown to engraft to high levels without the need for exogenous cytokines, and therefore provide an accessible assay system for very primitive human haematopoietic progenitor cells, and for testing the efficacy of gene transfer to these cells. In fact, while the majority of LTC-IC are readily transduced they may not be capable of long-term engraftment of SCID mice (Larochelle *et al.*, 1996). This would support the notion that SCID repopulating cells (SRC) are biologically distinct and more representative of human long-term repopulating cells, although the exact relationship between the SRC and the PHSC population has not yet been defined.

9.6 CONCLUSIONS

SCID remains one of the most attractive disorders for the application of somatic gene transfer technology. However, the clinical experience gathered to date highlights the fact that even for diseases in which complex gene regulatory systems are not necessary, the potential for efficacy may still be some way off. In common with other haematopoietic diseases, there is a need to optimise gene transfer and expression mechanisms in the laboratory. Development of animal models, both of human haematopoiesis and of specific disease phenotypes by gene targeting, is necessary to ensure that somatic gene transfer can provide clinical benefit.

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10 Gene Therapy for Haemophilia

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10.1 INTRODUCTION

Haemophilia is a congenital coagulation disorder that is characterized by uncontrolled haemorrhagic episodes that are crippling and potentially life threatening. Haemophilia A results from subnormal levels of an essential cofactor protein, factor VIII (F.VIII), and affects 1 in every 10 000 males; haemophilia B is associated with a lack of an essential protease, factor IX (F.IX), and occurs in 1 out of 50 000 males. Due to the absence of these key intermediates in the clotting cascade, haemorrhage is the most frequent cause of death in untreated haemophiliacs.

To date protein-replacement therapy is the treatment of choice. This treatment essentially normalizes both the life expectancy and the quality of life. Notwithstanding its tremendous achievements, this therapy has several drawbacks. The treated patient is still prone to spontaneous haemorrhages with the associated risk of chronic joint damage. In addition, therapy with plasma-derived F.VIII has resulted in transmission of several human viruses, such as HIV and hepatitis viruses. The risk of exposure to blood-borne pathogens has been virtually eliminated by improved manufacturing procedures and, more recently, by application of recombinant-DNA-derived F.VIII. Nevertheless, the ideal therapy would be independent of blood-derived products [1] and would provide a sustained therapeutic effect. Gene therapy may hold the promise of such a treatment of haemophilia and could, in theory, completely cure the disease.

10.2 GENE THERAPY FOR HAEMOPHILIA

10.2.1 PROPERTIES OF FACTOR VIII

The F.VIII protein is a large multimeric glycoprotein (~300 kDa) that circulates in plasma at low concentrations. The protein is synthesized mainly in the liver as a single-chain polypeptide, which by intracellular processing is converted into a two-chain dimer of 80 kDa and 200 kDa subunits. Before the actual activation of the F.VIII protein, a large segment of the 200 kDa subunit (the B domain), is removed, resulting in a 90 kDa heavy chain complexed to the 80 kDa light chain. Further proteolytic cleavage activates the F.VIII protein [2,3]. The F.VIII protein is translated from an mRNA of approximately 9000 nt, of which 7053 nt are coding. The F.VIII gene, located on the X chromosome, is about 186,000 base pairs (bp) in size (Figure 10.1).

Production of recombinant-DNA-derived F.VIII using the human F.VIII cDNA has been difficult. Firstly, the F.VIII cDNA has been found to contain sequences that repress its expression, resulting in low levels of F.VIII-specific mRNA [4,5]. Secondly, the majority of the F.VIII protein is transported inefficiently from the endoplasmic reticulum to the Golgi system due to retention of the protein in the endoplasmic reticulum [3]. Thirdly, the protein is extremely sensitive to proteolytic degradation and needs to be stabilized by von Willebrand factor. In addition, the protein undergoes extensive post-translational modification and needs to be proteolytically cleaved for its functional activation [2].

The F.IX protein is much smaller in size (55 kDa), and 500 times more abundant on a weight basis. Its gene is located on the X chromosome and is 33,000 bp in size. Whereas the F.VIII has no intrinsic enzymatic activity, the activated F.IX functions as a serine protease. It is secreted as an inactive precursor protein that can be activated by proteolytic cleavage. The F.IX protein is modified extensively. The first 12 glutamic acid residues of the Gla domain are γ -carboxylated post-translationally. This modification is essential for Ca^{2+} binding and F.IX function (see [6] for a review).

10.2.2 EX VIVO GENE THERAPY: PROBLEMS WITH RETROVIRAL VECTORS

Many studies have focused on the development of retroviral vectors for transfer of an F.VIII gene. In all studies published so far, F.VIII cDNA clones were used in which the non-essential B domain was removed. The sequences coding for the 90 kDa heavy chain were fused in-frame to the 80 kDa light chain codons. Removal of the B domain does not significantly affect any known function of the protein; the complete and the B-domain-deleted F.VIII

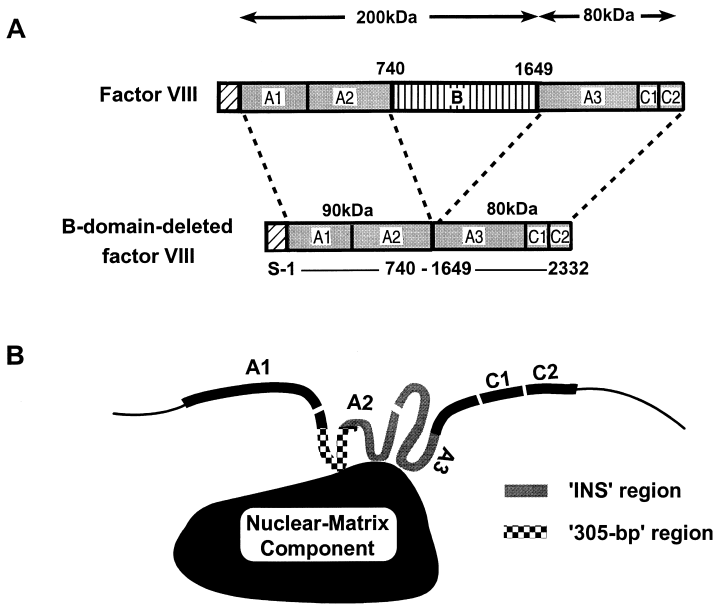


Figure 10.1. (A) Schematic representation of the domain structure of the F.VIII protein. The functional domains are organized in the order S-A1-A2-B-A3-C1-C2. S is the signal peptide. Upon secretion, the protein is cleaved at amino acid 1649 to generate a complex of the 200 kDa heavy chain (A1-A2-B) and the 80 kDa light chain (A3-C1-C2). Further cleavage at amino acid 740 removes the B domain from the heavy chain. In the B-domain-deleted form of the cDNA the sequences encoding the 90 kDa heavy chain are fused in-frame to the codons for the 80 kDa light chain. In some studies [8,13] a small spacer is left between the two chains. (B) Hypothetical model for the repression of transcription in the F.VIII cDNA. Data presented by Fallaux *et al.* [18] suggest that AT-rich elements similar to sequences found in the nuclear-matrix attachment regions (MAR) are involved in the repression of transcription of the F.VIII cDNA. MARs consists of modular A/T-rich sequence elements that act in concert to regulate the higher-order structure of chromosomal DNA in the nucleus. In this way MARs modulate expression of genes. In the F.VIII cDNA the presence of a number of multiple elements could form a functional MAR. Such a model can explain the difficulties in pinpointing the sequences involved in the repression. So far, there is no evidence to support any physiological relevance for the presence of the repressor sequences in the F.VIII cDNA. In the figure both the 1200 bp INS region identified and characterized by the Miller group [4,17] and the 305 bp region that is involved, but not sufficient for repression, are indicated [5,18].

variants are virtually identical in functional assays [7]. These B-domain-deleted cDNA clones have a size of approximately 4500 bp, and therefore can be inserted into retroviral vectors without exceeding the packaging capacity of the virus. Retrovirus-mediated transfer of the B-domain-deleted F.VIII cDNA has been achieved in various cell types, e.g. skin fibroblasts [8,9], endothelial cells [10–12], myoblasts [13], haematopoietic progenitor cells [14], intestinal epithelial cells [15] and bone marrow stromal cells [16]. The F.VIII secreted by these cells is functional, illustrating that cells of non-hepatic origin also have the capacity for proper post-translational modification of the F.VIII protein. This underscores the concept that gene therapy for haemophilia is not necessarily restricted to genetic modification of the hepatic cells that normally produce F.VIII.

In general, synthesis of F.VIII by genetically modified cells in culture has been quite low. Both the titre of the retroviral vectors and the amounts of F.VIII secreted by the transduced cells are reduced about 100-fold in comparison to those carrying F.IX and other cDNAs [4,5]. The low titres and the reduced amounts of F.VIII produced are caused, at least in part, by the very low amounts of F.VIII-specific transcripts that accumulate in the transduced cells [4]. There is now ample evidence that the inhibition of expression is caused by sequences in the F.VIII cDNA itself and that repression occurs at the level of transcription [4,5,17]. Lynch *et al.* [4] located a 1.2 kbp stretch of the F.VIII cDNA (INS) that reduces the titre of the F.VIII retroviral vectors. These sequences inhibit the F.VIII mRNA accumulation in the cytoplasm. In independent experiments we identified a 305 bp region in the F.VIII cDNA that is involved in the repression phenomenon [5]. Intriguingly, the latter fragment is juxtaposed to, but located outside, the INS region. In the 305 bp region, sequences were found that resemble the autonomously replicating sequence-consensus (ARSc) sequences of yeast, and the A/T-rich sequences found in mammalian matrix attachment regions (MAR). It has been shown that multimerization of the F.VIII cDNA-derived ARSc/MAR-like sequences could functionally mimic the repression phenomenon when linked to a heterologous reporter gene. Also, de-repression of expression by sodium butyrate could be mimicked using multimers of the F.VIII-derived sequences. This suggests that such ARSc/MAR-like sequences, dispersed throughout the F.VIII cDNA, may alter the chromosomal context of the F.VIII-expression vector (e.g. by associating to the nuclear matrix), resulting in repression of expression [18].

To improve the expression Chuah and co-workers [12] used a conservative mutagenesis strategy to introduce the maximum number of nucleotide changes in the 1200 bp INS region. Despite their impressive efforts, this increased neither the virus titre nor F.VIII expression. However, the insertion of an intron in their retroviral vector increased F.VIII expression up to 20-fold, and boosted virus titres up to 40-fold. This correlated with an

increase in mRNA accumulation, which suggests that the inclusion of an intron in the retroviral backbone relieved the transcriptional repression [12].

Although problematic expression has been found to occur with many retroviral vectors, some appear to be less prone to the inhibition. Dwarki and colleagues [11] reported F.VIII expression levels and vector titres that are at least 10- to 100-fold higher than those reported by others. In their vector, based on the MFG retroviral vector, the F.VIII cDNA is located at the exact position of the retrovirus *env* gene. Thus, the F.VIII message is translated from the spliced sub-genomic mRNA. Chuah and co-workers [16] exploited similar vectors. The efficiency of the system could be further boosted by optimizing the tissue-culture media and the infection procedures, resulting in a vector that is efficient enough to eliminate the requirement for a selection step prior to re-implantation of the modified cells [16]. Although the efficiency is not easily understood considering the repression that has been reported by others, these are the first F.VIII vectors that meet the requirements with respect to efficiency of a clinically applicable F.VIII retroviral vector.

10.2.3 IMPLANTATION OF RETROVIRALLY TRANSDUCED CELLS

Several cell types can be considered as targets for genetic modification in a protocol for gene therapy for haemophilia. Diploid skin fibroblasts are attractive targets. These cells can be harvested easily from patients, grown to large numbers in tissue culture and transduced with retroviral vectors with relative ease. In initial experiments F-VIII-secreting fibroblasts of murine or human origin, embedded in an artificial collagen matrix, were implanted subcutaneously on the midbacks of nude mice. In the case of human fibroblasts, cells isolated from the grafts 8 weeks after implantation still had the capacity to secrete F.VIII when regrown in culture. These results demonstrate the persistence of the transplanted cells in a metabolically active state [19]. Unfortunately, no human F.VIII could be detected in the recipients' plasma that might have been secreted by the implanted cells. This was attributed to the short half-life of the human F.VIII protein in mice. Dwarki and colleagues [11] observed circulating F.VIII after intravenous and intraperitoneal injection of recombinant F.VIII protein. In parallel experiments these authors could not detect human F.VIII following intramuscular or subcutaneous injection. This may be due to the susceptibility of the protein to proteolysis, resulting in degradation of F.VIII before it can reach the circulation. After intraperitoneal implantation of F.VIII-secreting fibroblasts into immunodeficient mice, circulating human F.VIII could be detected (maximally 100 ng/ml) in their plasma for up to 10 days [11]. The capacity of

transduced cells to deliver the F.VIII into the circulation was dependent on the site of implantation. These data convincingly demonstrate the feasibility of this approach, although the persistence of expression obviously needs to be increased.

10.2.4 *IN VIVO* GENE THERAPY: ENCOURAGING RESULTS WITH ADENOVIRAL VECTORS

Conceptually, protocols involving *in vivo* gene transfer are more straightforward than the *ex vivo* approaches. Connelly *et al.* [20] studied this approach using a recombinant adenoviral vector, Av1ALH81, in which the F.VIII cDNA is driven by a liver-specific mouse albumin promoter. The use of this vector circumvented many of the problems associated with retroviral vectors in *ex vivo* gene transfer strategies. HepG2 hepatoma cells transduced with Av1ALH81 secreted high levels of biologically active human F.VIII ($> 240 \text{ ng}/10^6 \text{ cells}/24 \text{ h}$). Administration of Av1ALH81 to mice resulted in an efficient transduction of the liver (the systemically administered adenovirus exhibits a strong hepatotropism). The resulting F.VIII levels in the recipients' plasma peaked at $300 \text{ ng}/\text{ml}$. These levels are even more impressive if one considers the short half-life of the human protein in mice. Normal F.VIII levels in humans are $100\text{--}200 \text{ ng}/\text{ml}$, and levels as low as $10 \text{ ng}/\text{ml}$ are therapeutic. Thus, the mice were producing human F.VIII at levels that exceeded those in normal human plasma. In the recipient mice F.VIII levels in plasma peaked at day 7, and decreased slowly to background levels 7 weeks after treatment. The decline in plasma F.VIII levels correlated with the loss of vector DNA from the liver. This is caused by elimination of the transduced hepatocytes by the host's immune system [21,22]. An optimized F.VIII adenoviral vector, Av1ALAPH81, was generated that carries an intron in the F.VIII expression cassette [23]. The F.VIII plasma levels (up to $2,000 \text{ ng}/\text{ml}$) in mice that received this vector exceeded those obtained with Av1ALH81. This allowed the administration of lower, less toxic, vector doses while maintaining sufficient levels of human F.VIII in the plasma of the recipient mice. F.VIII levels in plasma in the therapeutic range persisted for at least 22 weeks after a single administration of the vector [24]. In hemophiliac dogs, administration of this vector resulted in a complete correction of the bleeding tendency. However, a human F.VIII inhibitor antibody response developed. This resulted in elimination of the human F.VIII protein and reappearance of the hemophilia phenotype [25]. Thus it remains to be established whether in the large animal models for haemophilia A (e.g. haemophiliac dogs) and, ultimately, in humans, vector virus doses can be found that combine adequate and persistent F.VIII levels in plasma with the absence of hepatotoxicity and an F.VIII inhibitor response.

10.3 GENE THERAPY FOR HAEMOPHILIA B

The developments in the field of gene therapy for haemophilia B paralleled, and often preceded, those for haemophilia A. Starting in 1987, a variety of cultured cells have been transduced with retroviral F.IX vectors (reviewed in [26]). In general, functional F.IX was found to be secreted in significant amounts. However, transplantation of the transduced fibroblasts into mice resulted in transient F.IX plasma levels that were lower than would be expected on the basis of the F.IX secretion *in vitro* [27–31]. In some of the recipients the formation of F.IX inhibitors could be established, explaining the disappearance of circulating F.IX [31]. In addition, the retroviral long terminal repeat (LTR) promoter that drives expression of the gene of interest was found to be inactivated in fibroblasts *in vivo* [27–29]. Although the latter problem can be overcome by using a cellular promoter [30], such promoters are generally not very strong. Despite these problems, in 1993, Lu and colleagues initiated a phase I gene therapy trial with retrovirus-transduced autologous skin fibroblasts [32]. Two brothers with haemophilia B were treated. The authors report that in one patient, F.IX clotting activity increased significantly (from 2.9% to 6.3%) and persistently (over 6 months), but not in the other individual. Although encouraging, this trial is still a matter of debate [33].

The efficacy of *in vivo* gene therapy for haemophilia has been demonstrated by Kay and collaborators [34]. They infused F.IX retroviruses in haemophiliac dogs (beagles) that had previously undergone partial hepatectomy to stimulate the remaining hepatocytes to divide. Despite the low amounts of F.IX produced (approximately 0.1 % of normal), the average clotting time was reduced by approximately 60%. The production of the clotting factor persisted for over 9 months [34]. These results are very promising, although a further 10- to 100-fold increase in production is required to reach a clinically beneficial range.

Adenoviral vectors have also been used for the gene transfer of a human F.IX gene into mice. After a single intravenous dose into the tail vein, amounts of 400 ng/ml human F.IX could be detected in the recipient mice [35]. However, the levels slowly decreased to baseline within the course of 10 weeks. A second administration of the virus did not re-establish human F.IX plasma levels. This was due to high amounts of circulating antibodies that were generated, which neutralized the vector viruses upon rechallenge [35]. Similar results have been obtained in F.IX-deficient dogs [36]. After a single dose of the virus (administered into the portal vein) the bleeding tendency of these dogs was transiently corrected with an increase in F.IX levels from 0% to 300% of the level present in normal dogs. Although therapeutic levels could be maintained for 1–2 months, the F.IX levels decreased significantly in time. Nevertheless, these studies formally provide the long-awaited proof-of-principle.

Recently, vectors derived from the adeno-associated virus (AAV) have been used for the expression of F.IX in cultured cells [37] and *in vivo* [38–40]. With these vectors significant levels of F.IX protein could be observed in the recipient mice up to 5 months post-infection. Although the expression is still low, the AAV-derived vector's capacity to infect non-mitotic cells makes it an important alternative for the retroviral vectors, especially for *in vivo* liver-directed gene transfer.

10.4 WHERE DO WE GO TO FROM HERE?

Some of the hurdles on the road to gene therapy for haemophilia have been taken. The results obtained so far have demonstrated the potential efficacy and provided the conceptual proof-of-principle. However, several aspects need to be improved before clinical application can be considered for the treatment of haemophilia. In the *ex vivo* approaches the techniques for cell isolation, gene transfer and cell transplantation need further improvement. Also, the persistence of expression and the level of expression need to be enhanced. On the *in vivo* route it will be essential to efficiently target the gene-transfer vector to the desired tissue to ensure specific delivery of the curative gene into the cell type of choice. Ways must be found around the immune problems that restrict the applicability *in vivo* of the current adenovirus vectors [41]. It will be essential to limit the cellular immune response directed against the transduced cells. Also, the rapid humoral response, which generates neutralizing antibodies that inhibit subsequent virus-mediated gene transfer, reduces the applicability. Irrespective of the gene-transfer vector chosen and irrespective of the strategy followed, a major task will be to acquire the technology to appropriately regulate the expression of the healing gene.

These issues are not unique for haemophilia, but are imperative for all gene-therapy approaches for the treatment of congenital disorders. A concern that is more prominent in the case of haemophilia than in other disorders is the potential humoral response against the transgene product (F.VIII or F.IX). Such inhibitors, which are also formed in a minority of patients upon regular treatment, inhibit not only genetic therapy but also conventional replacement therapy. It needs to be established at what frequency inhibitors (F.VIII or F.IX antibodies) occur after gene therapy. To determine such frequencies, studies must employ homologous cDNA. The cloning of canine F.IX cDNA [42] and canine and murine F.VIII cDNAs [43,44] permits evaluation of gene-therapy procedures in canine and murine [45–48] models for haemophilia. This will allow a detailed comparison of current and future methods for haemophilia management with respect to safety and efficacy.

Notwithstanding the promising results, we should realize that gene therapy has only recently emerged as an approach for the treatment of various diseases. With the input from academic institutions and (biotech) industry steadily growing, the number of potential applications, too, is increasing. Applications are found for the treatment of AIDS, cancer, arthritis, Parkinson's disease and many hereditary diseases. Some of these applications have already reached the stage of phase I clinical trials.

With the increased input, the range of available tools is also expanding. New viral vector systems are being developed with improved applicability, yield and safety features. In addition, novel very efficient non-viral gene-transfer methods have been described that eventually may match and even surpass the efficiency of the viral vector systems. In this respect it is worth while to note how the viral and non-viral systems converge. On one hand, the safety of viral gene-transfer systems is further increased by reducing the content of virus (-derived) products in the vector. On the other hand, the non-viral vectors mimic the viral functions as much as possible using synthetic ingredients, resulting in artificial 'viroid-particles'. In this respect the work of Birnstiel's group is exemplary and has already been used for the expression of clotting F.VIII in mice [13]. It is therefore reasonable to anticipate that the future will hold promise of vector systems that can be administered systemically and that will target the gene of choice to a predetermined target tissue in a very efficient and highly specific manner.

In addition to these 'scientific' aspects we will need considerable efforts at the level of the production of the vectors. The type of therapeutics that is being considered for clinical application differs in several aspects from the more 'conventional' drugs. Hence at the production side, considerable investment needs to be made in order to acquire the technology to produce clinical-grade vectors in sufficient quantities.

Gene-therapy research thus requires the concerted action of scientists from many backgrounds, e.g. from fundamental research in virology, genetics and process technology to (pre-) clinical research in the fields of haematology, pediatrics and surgery. Once we have been able to solve the scientific and the technical problems, and only if we have unequivocally demonstrated the long-term safety and efficacy of this new technology, gene therapy can become a significant alternative to the current treatment of haemophilia.

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11 Cystic Fibrosis: Gene Therapy Approaches

NATASHA J. CAPLEN

11.1 INTRODUCTION

Cystic fibrosis (CF) has long been seen as a candidate for treatment by gene therapy. Reasons for this include: (i) the genetics of CF are straightforward, (ii) there is no long-term treatment for the disease, (iii) the lung, the principal organ affected by the disease, is accessible, and (iv) less than 100% gene transfer may still modulate the disease process. The identification and cloning of the affected gene in individuals with CF in 1989 rapidly led to clinical studies testing the feasibility of gene therapy for cystic fibrosis. This chapter reviews the mechanisms underlying the pathology of CF and the current status of gene therapy as applied to the disease.

11.2 THE GENETICS OF CYSTIC FIBROSIS

CF is a single gene disorder, inherited as an autosomal recessive characteristic. Within the Caucasian population approximately 1 person in 20 is a carrier of the abnormal gene and 1 in 2000 live births is an individual with CF. The affected gene is located on the long arm of chromosome 7 and is termed the cystic fibrosis transmembrane regulator (CFTR) gene (Rommens *et al.*, 1989). The genomic sequence covers approximately 250 kb, contains 27 exons, and encodes an mRNA of about 6.5 kb (Rommens *et al.*, 1989; Zielenski *et al.*, 1991). The most common mutation associated with CF is a 3 base pair deletion in exon 10 ($\Delta F508$), which accounts for approximately 70% of CF chromosomes (Kerem *et al.*, 1989). In total, over 400 CF-associated mutations have been identified (Tsui, 1992).

11.3 CFTR PROTEIN AND DISEASE PATHOLOGY

The *CFTR* gene encodes a protein which consists of two transmembrane regions, two nucleotide binding domains, and a regulatory region containing a number of potential phosphorylation sites (Riordan *et al.*, 1989). The CFTR protein is a cAMP-regulated chloride channel (Anderson *et al.*, 1991; Welsh *et al.*, 1992) found on the apical surface of epithelial cells of the conducting airways, intestine, pancreas, liver, sweat glands, and in males the vas deferens (for review see Cohn, 1994). Mutations in *CFTR* can be classified according to their effect on CFTR production and function. Most stop mutations result in no production of protein at all, whereas others, like the $\Delta F508$ mutation, produces protein that is unprocessed, never reaching the cell surface; both cause a significant reduction in chloride secretion. Two further classes of mutation result in abnormal channels that can secrete chloride, but do so in an unregulated manner or at a reduced level (Welsh and Smith, 1993).

The relationship between mutations in the *CFTR* gene, dysfunctional chloride secretion, and the pathology of CF is poorly understood and the subject of much debate. One theory suggests that under normal conditions chloride secretion on to the mucosal surface through CFTR allows water to follow by osmosis. This water movement maintains hydration of the epithelial cell surface and associated fluids. Within the gastrointestinal tract this dehydration of gut contents may account for the intestinal blockage seen perinatally in approximately 10% of CF patients and the plugging of ducts within the pancreas, and its subsequent atrophy, seen eventually in 80–90% of patients. In the lung, disrupted water movement may also result in a disturbance of mucociliary clearance (MCC), the process which ensures removal of inhaled particles and bacteria through the synchronized beating of cilia. With thickened mucus and inefficient MCC, the lung becomes subjected to repeated bacterial colonization. Recurrent infection with the bacterial strains *Staphylococcus aureus*, *Haemophilus influenzae*, *Pseudomonas aeruginosa* and *Burkholderia cepacia* contributes significantly to the cycles of inflammation that lead to bronchiectasis and respiratory failure in CF patients. Another factor is sodium absorption, which is also abnormal in CF patients, being increased 2- to 3-fold (Knowles *et al.*, 1981). However, since water will again follow sodium movement, this second abnormality will tend to dehydrate epithelial lining fluid and may thus further contribute to the thickening of mucus directly or the impairment of MCC.

More recently some studies have begun to suggest that there may be a more direct link between CFTR and bacterial adherence and/or colonization of the CF airway. One study has shown that transfer of the human *CFTR* cDNA to primary CF nasal cells significantly reduces the number of bound *P.*

aeruginosa associated with these cells compared to un-transfected or mock transfected CF cells (Davies *et al.*, 1997). Another study has demonstrated that normal airway surface fluid contains a bactericidal activity. CF airway lining fluid, however, does not appear to be able to induce killing of *P. aeruginosa* or *S. aureus*. Transfer of the *CFTR* cDNA to CF cells corrected this defect in killing, as did exposure of CF epithelia to a low salt concentration, suggesting that disrupted chloride and sodium transport directly influence this bactericidal activity (Smith *et al.*, 1996). Recently it has been shown that natural antibiotics (defensins) produced in the airway are salt-sensitive and thus may be inactivate in the airway of individuals with CF. One candidate defensin is β -defensin-1, which exhibits salt-dependent antimicrobial activity against *P. aeruginosa* (Goldman *et al.*, 1997a). However, most of these experiments have been conducted *in vitro* or *ex vivo*; recent analysis of the ion composition of airway surface liquid using direct *in vivo* sampling showed no significant differences between CF and normal subjects (Knowles *et al.*, 1997).

11.4 TREATMENTS FOR CYSTIC FIBROSIS

Current treatments for CF concentrate on elevating the symptoms and delaying the progression of the disease. The most common therapies include enzyme supplementation and diet to treat pancreatic dysfunction, and physiotherapy, antibiotics and anti-inflammatories to control the development of airway disease. Though there have been a number of studies attempting to modulate the electrophysiological defect underlying CF by using alternative sodium- or chloride-related pathways there has been little progress in the past few years, thus gene transfer has been seen as a very valid approach for the treatment of CF.

11.5 GENE TRANSFER SYSTEMS AND CYSTIC FIBROSIS

11.5.1 BACKGROUND

Though CF is a multi-organ disorder, it is the respiratory aspects of the disease that are associated with the greatest morbidity and mortality, thus the airway has been the principal target for CF gene therapy. An important consideration in the development of a gene therapy strategy for CF is the need to establish the minimum level of gene transfer that could still modulate disease progression. Carriers of CF are phenotypically normal and thus demonstrate that 50% of the normal level of *CFTR* expression is sufficient to prevent disease. Interestingly, both *in vitro* and *in vivo* studies suggest that

even less expression may be sufficient to normalize chloride secretion. Transduction of between 5% and 20% of CF cells grown as a complete monolayer shows total restoration of chloride conductance (Goldman *et al.*, 1995; Johnson *et al.*, 1992; Zabner *et al.*, 1994a) and CF transgenic mice expressing only 5% of the normal level of *Cftr* show disproportionately more chloride secretion (approximately 50% of wild type) than if the relationship were linear (Dorin *et al.*, 1996). However, it should be stressed that it is unclear how these findings relate to any likely clinical benefit, though in CF transgenic mice expressing only 5% of wild-type *Cftr* there is a significant reduction in intestinal disease (Dorin *et al.*, 1996). It should also be noted that correction of sodium hyperabsorption appears to require much higher levels of gene transfer, possibly up to 100% (Goldman *et al.*, 1995).

The converse problem, overexpression of *CFTR* or expression in sites where *CFTR* is not normally expressed, has been addressed in only a limited number of studies. However, the data from these is broadly encouraging. *In vitro*, Rosenfeld and co-workers have shown that increasing levels of *CFTR* protein do not induce levels of cAMP chloride secretion over a certain level (Rosenfeld *et al.*, 1994) and *in vivo* transgenic mice expression of both human and mouse *CFTR* in the lung showed no effect on lung weight, morphology, or overall growth (Whitsett *et al.*, 1992).

Most of the currently available gene-transfer systems have been used to mediate *CFTR* DNA transfer, with variable amounts of success. Only the biology of these systems directly relevant to CF gene therapy is described here; please refer to other chapters for further details of the basic science and vectorology of each system.

11.5.2 RETROVIRAL-MEDIATED GENE TRANSFER

The first reports of *in vitro* correction of the CF chloride channel defect used retroviral-mediated transfer of the human *CFTR* cDNA to CF cell lines (Drumm *et al.*, 1990; Rich *et al.*, 1990). Primary cultures of basal and secretory airway epithelial cells, including progenitor cells, can also be transduced by retroviral vectors (Halbert *et al.*, 1996). However, *in vivo* retroviruses have been considered to be unsuitable for mediation of gene transfer to the airway. This is because mammalian C-type retroviruses require cell proliferation for proviral integration and gene expression, and airway surface epithelial cells are terminally differentiated and thus non-dividing or slowly dividing. This supposition has, at least in part, been validated by the observation that retroviral-mediated gene transfer can be induced in airway cells *in vivo* but only in the presence of a damaged epithelium, which will stimulate proliferation and allow access to the underlying progenitor cells, which are more rapidly dividing (Halbert *et al.*, 1996). It has, though, been shown that the cells within the surface epithelium and the collecting ducts of submucosal

glands of the human lung can divide (Leigh *et al.*, 1995). In particular, this study showed that the damaged airway epithelium of CF patients shows enhanced proliferation, which may mean that *in vivo* retroviral-mediated DNA transfer to the airway may still be feasible. Finally, alternative retroviral vectors to those based on the standard mammalian C-type Moloney murine leukemia virus (MoMLV) have been developed that have the advantage of infecting non-dividing cells. These vectors are based on the human lentivirus HIV-1 (Naldini *et al.*, 1996). Goldman and co-workers have shown that a pseudotyped HIV vector transduced non-dividing human airway epithelial cells *in vitro* whereas a MoMLV retrovirus did not (Goldman *et al.*, 1997b). But, though a *CFTR*-expressing HIV vector successfully transduced undifferentiated human CF bronchial xenografts, the vector did not transduce xenografts when the epithelium was differentiated and thus further study will be required to determine whether this new vector system can be developed for applications relevant to CF gene therapy.

11.5.3 ADENOVIRAL-MEDIATED GENE TRANSFER

Adenovirus (Ad) is a particularly attractive vector for gene transfer to the airway as it naturally infects the respiratory epithelia. Adenoviral vectors carrying the *CFTR* cDNA have principally been developed from Ad-2 and Ad-5 adenoviruses of the subgenus C. *In vitro* studies examining adenoviral-mediated *CFTR* gene transfer include successful transduction of CF cell lines (Mittereder *et al.*, 1994), polarized epithelial monolayers derived from CF cell lines (Mittereder *et al.*, 1994; Rich *et al.*, 1993; Zabner *et al.*, 1994a), and freshly isolated human nasal and bronchial samples from CF patients (Rosenfeld *et al.*, 1994). Adenovirus was first shown to transfer the human *CFTR* cDNA *in vivo* by administration of an Ad-5-*CFTR* vector to the lungs of cotton rats (Rosenfeld *et al.*, 1992). *CFTR* mRNA was detected on day 1, with transgene expression sustained for up to 4 weeks. Human *CFTR* protein was found in airway epithelial cells 11–14 days post-instillation. Similar results have been obtained in other studies in cotton rats after both single (Yei *et al.*, 1994) and repetitive (Zabner *et al.*, 1994b) administration. Ad-mediated *CFTR* gene transfer into the nasal cavity of CF transgenic mice (Cfr^{m1UNC}) has also been studied. However, even with very high titers of Ad-*CFTR* little change was seen in chloride transport and no correction of the sodium defect was seen (Grubb *et al.*, 1994). In contrast, Yang and colleagues have detected *CFTR* protein expressed from a second generation *E2a*-deleted recombinant Ad carrying the *CFTR* cDNA after delivery to Cfr^{m1UNC} mice. Mice were instilled with 2×10^9 pfu (plaque forming units) of Ad-*CFTR*, resulting in the detection of *CFTR* in 80% of bronchi and for up to 21 days (Yang *et al.*, 1994). Ad-mediated transfer of the *CFTR* gene to primates has shown expression throughout the airways (Brody *et al.*, 1994;

Engelhardt *et al.*, 1993; Zabner *et al.*, 1994b). Most definable cell types within the lung can be transfected, including the surface epithelium of the trachea, bronchi and bronchioles, basal, mucous, goblet and ciliated cells, and sub-mucosal glands and alveolar (Bout *et al.*, 1994; Engelhardt *et al.*, 1993). It has been suggested that *in vivo* the susceptibility of different airway cells to Ad-5 varies, with columnar cells showing reduced susceptibility compared to basal cells; mechanical damage to remove columnar cells significantly enhances adenovirus transduction (Grubb *et al.*, 1994). These differences in transfection efficiencies may relate to the differential expression of the principal binding and internalization proteins, the $\alpha_v\beta_5$ integrins, utilized by adenovirus. These are expressed at high levels in undifferentiated cells and at very low levels in differentiated columnar cells (Goldman and Wilson, 1995). Another study has extended these findings by examination of patient tissue and has shown that the nose and trachea are void of $\alpha_v\beta_5$ integrin expression but that the integrin is present in the epithelial cells of the distal conducting airways (Goldman *et al.*, 1996).

In the first published clinical study of Ad-mediated *CFTR* gene transfer, Zabner *et al.* applied an Ad-2-based vector to the noses of three volunteers with CF. The nasal mucosa acts as a useful model for gene transfer to the lower airway because it is more accessible, and safety and efficacy can be assessed relatively easily. The nasal epithelium is contiguous with the lung and clearly demonstrates the characteristic ion transport abnormalities of CF. Measurements of both sodium and chloride transport can be assessed *in vivo* by measurements of potential difference (PD); all measurements reliably distinguish patients with CF from subjects without disease (Knowles *et al.*, 1995a; Middleton *et al.*, 1994). In the study by Zabner and co-workers a degree of localized inflammation around the site of application was seen, probably related to the method of delivery. *CFTR* mRNA was demonstrated in two subjects and changes in measurements of both sodium and chloride transport towards or into the normal range were detected in all three patients. These changes lasted for up to 10 days after the single application, although the study was not designed to assess duration of expression (Zabner *et al.*, 1993). The data from this small study are encouraging, but it should be noted that inflammation of the epithelium can influence some measurements of sodium transport and that subsequent trials investigating *CFTR* gene transfer to the nose have used a slightly revised methodology to assess the chloride response.

As part of an ongoing clinical protocol conducted by Crystal and co-workers in which patients receive an Ad-5-*CFTR* vector to the nasal epithelium, *CFTR* mRNA was observed in a sample from one of four patients presented so far and protein in another (Crystal *et al.*, 1994). Study of the bioelectrical characteristics of nine patients showed an improvement in chloride secretion, towards normal values, of approximately 30% over a

2-week period after administration; there was no evidence of vector-induced epithelial damage in any patient (Hay *et al.*, 1995).

In a third trial, which unlike the previously described ones was blinded and placebo controlled, 12 CF patients were recruited to receive one of four different doses of adenovirus or placebo to the nasal epithelium (Boucher *et al.*, 1994). Adenovirus-derived *CFTR* mRNA was demonstrated in five of six patients who received the highest doses of vector. However, no consistent changes in chloride or sodium transport measurements were found. At the lower doses of vector, no toxic effects were observed, but at the highest dose (a titer of 2×10^{10} , MOI of 1000) there was mucosal inflammation in two of the three patients (Knowles *et al.*, 1995b).

Trials have also been initiated assessing the effect of Ad-*CFTR* delivery to the lung. As well as applying the adenovirus to the nose, Crystal and co-workers have administered an Ad-*CFTR* vector to the lower respiratory tract by instillation through a bronchoscope. One bronchial sample was found to be positive for normal *CFTR* protein (Crystal *et al.*, 1994). However, one of the patients (who received the highest dose, 1×10^{10} pfu) developed hypotension, fever, and respiratory symptoms suggestive of an inflammatory reaction within the lungs. The investigators have suggested that these changes may relate to an increase in interleukin-6 (IL-6) as a direct result of the adenoviral infection. All clinical symptoms of this event resolved completely over a period of 1 month (McElvaney and Crystal, 1995). Similar results have been observed in another ongoing lung trial. In this protocol (Dorkin, 1998a), up to 2×10^9 infectious units (IU) of an Ad-2-based *CFTR* vector was delivered by bronchoscopy; *CFTR* mRNA was detected at higher doses but variable localized inflammation was also seen in most patients. In contrast, in the second arm of this study (Dorkin, 1998b), where the Ad vector is being delivered by aerosolization, vector-derived *CFTR* mRNA has been detected but no toxicity has been observed (David Meeker, personal communication). Data from another trial assessing aerosolized delivery of an Ad-*CFTR* vector has also shown no obvious toxicity (Bellon *et al.*, 1997).

Clinical trials assessing the effect of repeat administration of Ad-*CFTR* have also begun. In an extension of their first CF gene therapy trial, Zabner and co-workers investigated repeated administration of an Ad-*CFTR* vector (Ad-2-*CFTR2*) to the nasal epithelium (Zabner *et al.*, 1996). Six patients received four or five increasing doses (from 2×10^7 IU to 1×10^{10} IU) of an Ad-*CFTR* vector (Ad-2-*CFTR2*) at least 28 days apart. Only one nostril was treated so that the second could be used as a control to determine the effect of *CFTR* gene transfer. Though no overall statistical difference could be detected between the treated and untreated nostrils, individual patients did show some indications of alterations in their response to stimulation of chloride secretion following Ad-2-*CFTR2* administration. These changes appeared to be maximal at doses of 2×10^9 and 6×10^9 , with little or no effect at 1×10^{10} .

This reduction in the correction of chloride transport was paralleled by evidence of an immune response to adenovirus at doses of 2×10^9 and higher. The exact nature of this immune response was variable but in some patients was detected as a transient increase in serum IgG, in others as an increase in serum-neutralizing antibodies, and in others as a change in the antibody response to specific Ad proteins, including the recognition of new epitopes. The authors thus speculate that the reduction in *CFTR* correction is a result of humoral immunity to adenovirus blocking subsequent infections by the vector. However, it should be noted that preliminary data from another trial assessing repeated administration of a Ad-*CFTR* vector, but this time directly into the lung, has seen no evidence of an increased antibody-associated immune response to the vector following a second or third application of the adenovirus (Harvey *et al.*, 1997).

11.5.4 ADENO-ASSOCIATED-VIRAL-MEDIATED GENE TRANSFER

Adeno-associated virus (AAV) is a single-stranded DNA virus, which has the potential to integrate into the host genome, often in the same location on chromosome 19. Helper viruses such as adenovirus or herpes virus are required for productive viral infection, thus AAV is naturally defective for replication. The maximum packaging capacity of AAV vectors is approximately 4.5–5 kb, just allowing insertion and packaging of the *CFTR* cDNA (Flotte *et al.*, 1992). Vectors are constructed by removal of one or both of two genes, *rep* and *cap*. However, removal of some of the REP proteins appears to remove the preferential integration to chromosome 19 so that AAV vectors are normally found to persist in an episomal form (Afione *et al.*, 1996; Kearns *et al.*, 1996). AAV-*CFTR* vectors have been used to transduce both CF cell lines (Egan *et al.*, 1992) and primary tissue (CF nasal polyps) *in vitro* (Flotte *et al.*, 1993). Exogenous *CFTR* RNA and *CFTR* protein were detectable for up to 6 months in rabbits into which an AAV-*CFTR* construct had been instilled (Flotte *et al.*, 1993). The same group has also reported the presence of AAV vector DNA in airway epithelial cells obtained from rhesus macaques instilled with an AAV-*CFTR* vector 3 months previously (Conrad *et al.*, 1996); no adverse effects were detected using doses of virus up to 1×10^{11} total particles. Clinical trials of AAV-*CFTR* gene transfer are currently ongoing. In one study between 30 000 and 10 000 replication units (ru) of AAV are being administered to one nostril (the other nostril receives placebo) and 30 000 to 100 000 ru to the lung by bronchoscopy (Flotte *et al.*, 1996). Preliminary data has suggested the evidence of DNA transfer. However, full efficacy and safety data are still awaited. Other trials of AAV-*CFTR* transfer using the maxillary sinus as the target tissue have also begun (Gardner, 1998a,b).

11.5.5 CATIONIC LIPID-MEDIATED GENE TRANSFER

Cationic liposome-mediated DNA transfer utilizes a charge interaction between DNA (usually in the form of a plasmid), which is negatively charged, and a mixture of lipids (usually cationic and neutral) to form a complex of lipid-coated DNA. The exact nature of this interaction is unknown and may well be different for different cationic lipids. The mechanism of cell uptake and trafficking of these complexes through the cell is also poorly understood. Transfer of *CFTR* to HeLa and COS-7 cells using the cationic lipids DOTMA and DOTAP, respectively, has been shown to generate cAMP-specific chloride secretion in these cell lines (Hyde *et al.*, 1993; McLachlan *et al.*, 1995), and CF nasal cells obtained by brushing have been transfected *in vitro* using both DC-Chol:DOPE and DOTAP (McLachlan *et al.*, 1996; Stern *et al.*, 1995).

In vivo, delivery of *CFTR* plasmid DNA complexed with the liposome DOTMA:DOPE by intratracheal administration to mice showed human-specific *CFTR* mRNA transcripts present in the lung for up to four weeks (Yoshimura *et al.*, 1992). Three different cationic liposomes have been used to deliver *CFTR*-containing plasmids to CF transgenic mice. Hyde and co-workers (Hyde *et al.*, 1993) instilled a cationic liposome (DOTMA:DOPE) complexed with a *CFTR* cDNA into the trachea of *Cftr*^{m1Cam} transgenic mice and showed restoration of cAMP-stimulated chloride secretion. Nebulized DC-Chol:DOPE *CFTR* cDNA complexes delivered to *Cftr*^{m1HGU} mice showed correction of the CF chloride defect in the trachea and to a lesser extent in the nose (Alton *et al.*, 1993), as did direct application to the nose and tracheal instillation of the same transgenic mice with *CFTR* plasmid DNA complexed with DOTAP (McLachlan *et al.*, 1996). In a similar study using the cationic liposome DMRIE-DOPE, human *CFTR* mRNA was detected in rats instilled three days previously (Logan *et al.*, 1995). In most cases the presence of endogenous *CFTR* function cannot be distinguished from functional expression of the *CFTR* transgene. However, in the rat cAMP-mediated chloride conductance is minimal. Thus, Logan and co-workers also measured the ion transport characteristics of excised rat tracheas. Bioelectrical responses consistent with *CFTR* transfer were observed, including cAMP-mediated chloride secretion three days after instillation.

Several clinical trials of administration of DNA-liposome complexes to the nasal epithelium of CF subjects have now been reported. In the first 15 $\Delta F508$ homozygous subjects with CF were enrolled in a double-blind, placebo-controlled trial (nine *CFTR* treated, receiving either 20, 200 or 600 μ g DNA, and six placebo) (Caplen *et al.*, 1995). No safety problems were encountered, either in the routine clinical assessment or by a blinded, semi-quantitative analysis of nasal biopsies. Both plasmid DNA and *CFTR* mRNA were detected from the nasal biopsies in five of eight samples available from *CFTR*-treated subjects. Sodium-related measurements were significantly reduced

(approximately 20% toward values seen in patients without CF). However, it is important to note that these changes fell within the coefficient of variation of these measurements. More importantly, chloride secretion, assessed by perfusion with a low chloride solution, also showed a significant 20% increase toward normal values, a change well outside the variation in these measurements. In two subjects, these chloride responses reached values within the range seen in subjects without CF. These changes in the sodium- and chloride-related measurements paralleled each other, and were no longer present at seven days.

The trial performed by Gill and colleagues also used the cationic liposome DC-Chol:DOPE, but in a slightly different formulation and with the *CFTR* cDNA driven by the RSV long terminal repeat (LTR) (Gill *et al.*, 1997). In this study 12 CF subjects were randomized to either one of two doses of *CFTR* cDNA (40 and 400 μ g) or placebo. No safety problems were encountered. Using analysis of nasal brushings taken five days after DNA/liposome administration and *in vivo* PD measurements taken at regular intervals, of the eight *CFTR*-treated subjects, six showed changes consistent with restoration of chloride secretion. In most cases the level of correction was partial; however, in two patients (one from each dosing group) the chloride responses were within the non-CF range. Interestingly, these changes were more sustained than in the previously reported trial, lasting for approximately seven days. This difference may reflect the effect of using the RSV LTR promoter as against the SV40 early promoter which was used to drive *CFTR* expression in the first trial.

In another protocol the cationic lipid used was DOTAP (Boehringer Mannheim, Mannheim, Germany) and the *CFTR* cDNA was driven by the CMV promoter (Porteous *et al.*, 1997). Sixteen CF subjects were studied (eight treated, 400 μ g, and eight placebo). Again, no safety problems were encountered. Vector DNA was detected in seven out of eight patients at day 3 and day 7 and two patients at day 28. Vector-derived mRNA was detected in two patients at day 3 and 7. While there was much variability in the ion transport measurements observed, two patients showed changes in chloride conductance consistent with correction, with an average change towards normal of 20%.

Recently, new cationic liposomes specifically developed for gene therapy have been described (Lee *et al.*, 1996; Wheeler *et al.*, 1996). One of the most promising of these second-generation liposomes, GL67 (Genzyme Corp., Framingham, MA, USA), is currently being assessed. This lipid was first tested in a nasal study in which GL67-complexed *CFTR* plasmid DNA was administered to one nostril and *CFTR* plasmid DNA alone to the other nostril. Interestingly, though a significant correction in the chloride response was seen, no clear difference was seen between the two treatments, with DNA being as effective as DNA plus lipid (Zabner *et al.*, 1997). An advantage

to GL67 as a DNA delivery agent, though, has been that formulations of this cationic lipid have been developed that allow aerosolized delivery to the airway (Chadwick *et al.*, 1997; Eastman *et al.*, 1997, 1998). A clinical trial assessing the effectiveness of this aerosolized formulation in delivering *CFTR* cDNA to the lungs of CF patients has just been completed. The full results of this study will be published shortly (Dr E. Alton, personal communication) but in summary the study, performed as a collaboration between the National Heart and Lung Institute/Royal Brompton Hospital, London, UK and Genzyme Corp., showed in the lower airways on average a 25% correction of the basic CF defect in the majority of subjects (Alton *et al.*, 1998). It should be noted, though, that the DNA-lipid treated subjects showed a 'mild flu-like response over a period of approximately 24 hours' (Alton *et al.*, 1998). The investigators speculate that this may relate to the lack of methylation of DNA derived from bacteria which may induce an inflammation response. It is unclear what significance this will have for the long-term use of cationic liposome DNA transfer to CF gene therapy but as yet no problems have been reported following repeated administration of DNA-liposome complexes to CF transgenic mice. In this study the degree of correction of chloride secretion seen after two doses of *CFTR*/DC-Chol:DOPE, 10 days apart, was not significantly different from that seen after a single dose (Goddard *et al.*, 1997). A clinical trial assessing three doses of DNA-liposome complexes in CF patients, administered 28 days apart, has been conducted by the same group. Preliminary safety data has shown no significant clinical or immunological problems (Southern *et al.*, 1997). Finally, two other trials using different cationic liposomes to deliver the *CFTR* cDNA to CF patients have either been initiated or will be shortly (Knowles *et al.*, 1998; Sorscher, 1998; Sorscher *et al.*, 1994).

11.5.6 OTHER GENE TRANSFER SYSTEMS

Only limited studies have been conducted examining alternative gene transfer systems. However, in the long term these may prove to be applicable to the treatment of CF. A promising approach has been the use of specific receptors to mediate gene transfer. DNA is bound to an appropriate ligand which will interact with a receptor expressed on the cell surface. Several receptors have been used but the most studied that shows uptake by respiratory epithelial cells is the polymeric immunoglobulin receptor, which makes use of Fab antibodies to secretory component (Ferkol *et al.*, 1993). Uptake is through the basolateral surfaces of epithelial cells and thus DNA-ligand conjugates are administered intravenously. While DNA transfer has been successfully achieved in both the lung and liver, expression is transient, lasting about seven days (Ferkol *et al.*, 1995), and repeat administration results in a significant reduction in gene expression (Ferkol *et al.*, 1996). This

reduction is as a result of a response against the antibody component of the conjugate and thus alterations may be required to reduce the antigenicity of the antibodies used.

Two approaches are addressing a more permanent approach to gene therapy. The first is the construction of artificial chromosomes. This approach would make use of the *CFTR* genomic sequence within a construct which would contain the essential components of a chromosome, such as a centromere and telomeres. As yet the application of such constructs for gene therapy is only theoretical but early studies using *CFTR*-containing yeast artificial chromosomes are encouraging (Mogayzel *et al.*, 1997). Finally, one study has attempted to specifically alter the $\Delta F508$ mutation using recombination. This strategy makes use of small fragments homologous to the wild-type sequence in which the $\Delta F508$ mutation is found. *In vitro*, molecular and functional analysis showed successful gene targeting. However, the frequency of homologous recombination was estimated to be approximately 10^{-5} to 10^{-6} . It is likely that this frequency will need to be significantly enhanced if this approach is to be more widely applicable (Kunzelmann *et al.*, 1996).

11.6 GENE TRANSFER AND CF: WILL IT WORK?

Can we draw any conclusions as to how useful any of these systems are likely to be in the actual treatment of CF, particularly with respect to those that have now been tested in early clinical trials? The results of the many studies assessing Ad-mediated DNA transfer have thrown up some interesting problems, primarily related to the host response to the virus. Both in preclinical and clinical studies, a dose- and time-dependent inflammation has been observed after topical administration of Ad vectors to the airway (Brody *et al.*, 1994; Crystal *et al.*, 1994; Engelhardt *et al.*, 1993; Knowles *et al.*, 1995b; Simon *et al.*, 1993; Yei *et al.*, 1994). The inflammation seen in these studies appears to be related to the antigenicity of the adenoviral coat proteins, which induces a cytotoxic T lymphocyte response leading to destruction of virus-infected cells and thus loss of transgene expression (Yang *et al.*, 1995a). These studies have led to the construction of second-generation viruses with an inactivation of *E2a*. These vectors are associated with substantially longer recombinant gene expression and less inflammation (Engelhardt *et al.*, 1994; Yang *et al.*, 1994). Alternatively, immunosuppressive drugs such as cyclosporin and dexamethasone, which downregulate the immune response, have also been shown to lengthen exogenous expression in the airways of cotton rats (Zsengeller *et al.*, 1995). However, the most significant limit to the use of adenovirus may be that of the production of neutralizing

antibodies, which results in reduced gene transfer following a second administration of virus (Kozarsky *et al.*, 1994; Smith *et al.*, 1993; Zabner *et al.*, 1996). Studies in transgenic mice have shown that T cells contribute significantly to the formation of neutralizing antibodies in the airway which block subsequent adenovirus-mediated gene transfer (Yang *et al.*, 1995a,b). Thus, to overcome the problem of repeated administration additional immunological modulation may be required. For example, interleukin-12, if administered at the same time as the first administration of Ad, specifically blocks IgA production and results in a 20-fold reduction in neutralizing antibody titer and a maintenance in gene expression after re-administration of vector (Yang *et al.*, 1995b).

The major problem associated with cationic liposome-mediated gene transfer to the airway is that of efficiency. Currently, on a per molecule of DNA basis, viral vectors are several orders of magnitude more efficient than liposomes. It is unclear at what point in the transfer process the majority of DNA is lost following liposome delivery. Preliminary studies, however, suggest that the greatest barriers are the endocytic pathway and the nuclear membrane (Zabner *et al.*, 1995). Co-administration with pharmacological agents may thus be necessary to overcome these problems. An additional issue, which has yet to be systematically studied, is the problem of repeat administration of cationic liposomes. Though gene expression is maintained following repeated administration of DNA-liposome complexes to the airway (Goddard *et al.*, 1997; Scheule *et al.*, 1997), it is unclear what will be the long term effect on the delicate architecture of the lung of multiple exposures to large amounts of lipid.

Finally, clinical issues specific to CF must be addressed. Within the lower airways of patients with CF there are large quantities of infected secretions, as well as endogenous and potentially exogenous nucleases which may markedly alter gene transfer efficiency. *In vitro* studies have shown that mucus does indeed block DNA-liposome and adenoviral gene transfer. However, the use of mucolytic agents such as DNase can significantly reduce this inhibitory effect (Stern *et al.*, 1998). There is also the need to find additional outcome measurements to assess the success of gene therapy. At present *in vivo* bioelectrical techniques are well established only for measurements of nasal potential difference. Lower airway PD measurements are technically more difficult. However, preliminary data does suggest that they are feasible in CF patients (Alton *et al.*, 1996). More clinically relevant end-points may also be required. For example, measures of bacterial adherence, changes in mucus rheology or MCC, and radiological scans may need to be included in future protocols. Analysis of outcome measurements of this type will be essential in the future as gene therapy for CF moves from the 'proof of principle' stage described here to its establishment as a potential treatment.

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12 Therapeutic Approaches to Haemoglobinopathies

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12.1 INTRODUCTION

Haemoglobin disorders represent models for several gene defects, including deficiency in gene expression (thalassaemia syndromes), protein variant gaining a new function (the polymerisation of haemoglobin S in sickle cell disease) and de-repression of genes (hereditary persistence of foetal haemoglobin).

β Thalassaemia and sickle cell disease were the initial targets for gene therapy for several reasons, including the following:

1. The β globin gene is small, less than 2 kb. It is well expressed after transfer in transgenic mice, at the same level as the mouse globin genes, and specifically in the erythroid lineage, at the expected differentiation stage and without adverse effect in mice (Stamatoyannopoulos and Nienhuis, 1994).
2. The target, the haematopoietic stem cell with long-term repopulating activity *in vivo*, can be obtained from blood or bone marrow, genetically modified using an *ex vivo* process and grafted.
3. Relevant mouse models are available for β thalassaemia and sickle cell disease.
4. In 1986, β thalassaemic mice were cured by the human globin gene transferred to the germ line (Constantini *et al.*, 1986).
5. Detailed knowledge of regulatory sequences exists.
6. Molecular defects of haemoglobinopathies affecting all steps from gene to protein are well characterised and can be used for different strategies aiming at the addition, substitution or correction of globin genes.

Table 12.1 Therapeutic approaches to haemoglobinopathies at the gene or RNA levels

<i>β Globin locus</i>
Gene addition or replacement (1, 2)
Correction of a mutation (1, 2)
<i>γ</i> Gene activation (1, 2)
<i>α Globin locus</i>
<i>α</i> Gene inactivation (1)
<i>α</i> LCR inactivation (1)
<i>Globin RNA</i>
<i>β</i> Transcript: splicing correction (1)
<i>α</i> Antisense, ribozyme (1)
<i>Other genes</i>
Erythropoietin gene addition (1)
Red cell enzymes
2,3-biphosphoglycerate mutase (antisense) (2)
2,3-biphosphoglycerate phosphatase (gene addition) (2)
Anti-oxidising enzymes (1)

1, *β* Thalasassaemia; 2, sickle cell disease.

The activation of the *γ* foetal haemoglobin genes, in order to compensate the *β* globin gene defects, or the reduction of the deleterious effects of the mutation on the cellular phenotype are strategies of indirect gene therapies (Table 12.1).

The direct gene therapy of haemoglobinopathies began, rather prematurely, in 1980 with the addition of the normal human *β* globin gene to cultured bone marrow cells of two *β* thalassaemic patients followed by the injection of the manipulated cells into the patients. The results were negative and, it is hoped, not harmful to the patients. After this initial failure, success seemed to be at hand with the use of a cellular ‘Trojan horse’, a mouse retroviral vector transferring and expressing the globin gene in murine erythroleukaemia (MEL) cells in culture.

However, in spite of the many potential benefits of globin gene transfer, the haemoglobinopathies were no longer at the top of the list for potential gene therapy, because the *in vivo* expression of the somatic globin transgene was too low to reach the therapeutic threshold and was restricted to a small proportion of cells, which disappeared *in vivo* during long-term follow-up (Table 12.2). Basic studies were undertaken to improve the efficiency of gene transfer, the expression of the somatic globin transgene, and the number of haematopoietic stem cells available for gene transfer.

Table 12.2 Globin gene therapy

Advantages	Disadvantages
Small size of the gene (< 2 kb)	Requirement of genomic DNA
Good cellular and animal models	Dispersed regulatory elements
Cure of mouse β thalassaemia by human globin gene transfer into germ line	High expression of gene required upon terminal differentiation
Well known pathophysiology of the diseases	Quiescence and low proportion of target stem cells
	Requirement of large number of genetically modified cells and long-term expression of transgene <i>in vivo</i>

12.2 GLOBIN RETROVIRAL VECTOR

The initial Moloney mouse leukaemia retroviral vector (MoMLV) was efficient for the transfer of genes of interest in mouse haematopoietic cells *in vitro*. However, such retroviral vectors have a small capacity, less than 10 kb, for insertion of genes of interest, requiring selection of core regulatory sequences, present in the locus control region (LCR) of the β globin gene cluster, to be added to β globin genomic DNA. The retroviral vector requires the division of target cells for integration in the cellular genome. The stem cells with long-term repopulating activity *in vivo* are quiescent. Current studies are designed to increase the number of stem cells *ex vivo*, to induce cell cycling without generating their differentiation, to improve packaging cell lines to produce high titres of vectors having a potent transducing capacity for the human stem cell and to maintain high levels of transgene expression.

For high levels of expression and, erythroid specificity, the genomic globin gene must be used in inverse orientation, under the control of the globin promoter, associated to LCR hypersensitive sites (HS). However, frequent rearrangements and aberrant splicing sites or poly A termination sites are generated by the inverse orientation, requiring site-directed mutagenesis to improve both the vector stability and the expression of normal globin gene in target cells (Leboulch *et al.*, 1994; Sadelain *et al.*, 1995a).

MEL cells were used to study the expression of a single copy of the transduced globin gene, contrasting with concatamers induced by DNA transfection. Early studies have shown variable expression of the single copy provirus, related to the random integration site (Bender *et al.*, 1989), when only the proximal *cis*-active regulatory elements were present. The level of globin transgene expression was low in transduced MEL cells in comparison to the endogenous mouse globin genes (Bender *et al.*, 1989; Dzierzak *et al.*, 1988; Karlsson *et al.*, 1987; Kasahara *et al.*, 1994; Leboulch *et al.*, 1995). *In vivo*, the sustained expression of the human globin transgene was found infre-

quently, indicating that the transduction of long-term repopulating stem cells was rarely obtained. The expression of the β transgene was less than 5% of the endogenous mouse β globin genes.

The discovery of the HS of the remote LCR stimulated studies to determine which LCR site, or combination of sites, was most effective for globin gene expression. The limited size capacity of retroviral vectors for foreign transgenes caused the LCR region to be restricted from 20 kb to small core DNA fragments, rich in transacting motifs. The HS2 site of LCR was the most active (Caterina *et al.*, 1991; Collis *et al.*, 1990; Curtin *et al.*, 1989; Forrester *et al.*, 1989; Grosveld *et al.*, 1987; Leboulch *et al.*, 1995; Philippsen *et al.* 1990; Ryan *et al.*, 1989; Talbot *et al.* 1990). Ecotropic retroviral vectors containing truncated HS sites linked to the human β globin promoter yielded 60–70% expression in MEL cells compared with endogenous murine globin expression. Infection of mouse bone marrow cells and the transplantation of transduced cells into lethally irradiated recipients resulted in variable β globin transgene expression, higher than without the LCR cassette, but below the required threshold for therapeutic benefit (Chang, *et al.*, 1992; Gelinis, *et al.*, 1992; Novak, *et al.*, 1990; Plavec, *et al.*, 1993; Ellis *et al.*, 1997). Unfortunately, the new constructs generated instability, rearrangements of the provirus and low titres of vector.

Genetic instability was prevented by a number of systematic deletions and site-directed substitution (Leboulch *et al.*, 1994; Sadelain, *et al.*, 1995a). The resulting improved stability and viral titres (10^6 per millilitre) permitted the analysis of the effects of various β LCR cassettes in erythroid cells *in vitro* and *in vivo*. The expression of the β globin transgene associated with constructs containing four LCR sites averaged 78% of the murine genes and ranged from 4% to 146% (Sadelain, *et al.*, 1995b). In spite of the presence of the most active LCR sites, the expression of the somatic β globin transgene remains position dependent. The addition of 'insulator sites' at the ends of the β globin gene LCR construct may decrease the variability of the β globin gene expression (Chung *et al.*, 1993) and may be useful for avoiding activation of an oncogene near the insertion site of the provirus. The long-term expression of the stable β globin gene LCR construct, after transduction of the stem cells and transplantation, regrettably cannot yet be maintained (Sadelain *et al.*, 1995b).

These studies improved the design of retroviral vectors and have solved three major problems of β globin gene transfer with retroviral vectors: the generation of high titres of vectors, the stability of the transduced genome and high levels of β globin gene expression *in vitro*, in an erythroid-specific manner (Leboulch *et al.*, 1995).

The need for vectors capable of infecting human stem cells requires amphotropic or human packaging cell lines, not associated without reduction in the vector titre or reduced transduction capability. In mice, the expression of the human adenosine deaminase (ADA) cDNA under retroviral long ter-

minal repeat (LTR) control reached 80% of endogenous ADA at 12–14 months after transplantation, corresponding to the life-span of the transplanted animals (Rivière *et al.*, 1995). Specific features of viral LTR contributed to the increased duration and expression of the ADA gene in most cell lineages derived from the long-term repopulating stem cells.

12.3 ADENO-ASSOCIATED VIRAL VECTORS

The adeno-associated virus (AAV) is a single-stranded DNA virus, 4675 nucleotides in length, from the parvovirus family. AAV is nonpathogenic. It can infect a large range of quiescent human virus cells, and it can be integrated into a specific site on human chromosome 19 (19q13.3-qter) (Flotte *et al.*, 1995; Kotin *et al.*, 1991).

Sequences required for packaging, integration and replication are located within 191 nucleotides, which include the inverted terminal repeats (ITR) (Samulski *et al.*, 1989). AAV is a defective virus requiring co-infection with a helper virus, (adenovirus or herpes simplex virus) for efficient replication. Anti-AAV antibodies are present in 70–80% of the population.

AAV type 2 has been used as a viral vector to express a variety of transgenes. The maximum size of the packaged DNA is only 4.5 kb. Most of the genomic DNA can be replaced by a gene of interest because the AAV termini (145 nucleotides) are sufficient for integration of the recombinant AAV vector into the host genome.

The problem of contamination of the AAV vector by helper viruses used for complementation, required to produced infectious vectors, has been solved by using heat treatment, purification steps and the use of helper plasmids.

However, several problems remain unsolved: (i) the difficulty of obtaining a packaging cell line able to produce a high titre of AAV vectors, (ii) reproducible production of AAV vectors, (iii) the toxicity of Rep proteins for packaging cells and (iv) recombinant AAV vectors containing only the AAV termini may have lost the specificity for the chromosome 19 integration site (Mamounas *et al.*, 1995; Shelling and Smith, 1994).

Infection of cells of the human erythroid cell line K562 or primary human haematopoietic cells with the A γ globin AAV vector is associated with a high frequency of cells harbouring and expressing the A γ globin transgene (2.2 kb) marked with a 6 nucleotide deletion in the 5' untranslated region, linked to LCR site 2 (0.4 kb). K562 cells expressed the γ transgene at the level of the γ endogene in cells induced for erythroid differentiation (Miller *et al.*, 1994; Walsh *et al.*, 1992). CD34 + human haematopoietic cells exposed to A γ AAV vectors at a multiplicity of infection of 500–1000 particles per cell resulted in 20–40% of erythroid burst colonies expressing the A γ transgene at levels

4–71% that of endogenous γ genes. The foetal haemoglobin (Hb F) content of pooled colonies increased from 26% to 40% after AAV transduction in progenitor cells (Miller *et al.*, 1993). Rearrangement of the vector genome with loss of regulatory sequences may have occurred in a proportion of clones (Miller *et al.*, 1994). Alternatively, residual vector DNA gave an artificially false signal of DNA from some colonies (38), requiring RT-PCR for estimating AAV transduction efficiency and transgene expression. The integration of A γ AAV vector in erythroid colonies was not formally demonstrated. However, the high number of cells (> 1000) derived from a single transduced progenitor is consistent with proviral integration in the clonogenic cell.

Recent studies using the marked A γ globin gene, HS sites 2,3 and 4 from the LCR, but lacking a drug resistance gene, showed tandem integration and functional expression only at high multiplicity of infection of AAV vector (Hargrove *et al.*, 1997). Long-term expression of human γ globin gene was observed in primary transplants at a level up to 6% of the endogenous β globin gene expression for 1 month and 0.4% in bone marrow cells from secondary recipients (Ponnazhagan *et al.*, 1997).

Expression of the α globin antisense RNA in K562 cells, using a recombinant AAV 2 based vector, is an indirect approach to overcome the β thalassaemia phenotype (Ponnazhagan *et al.*, 1994) (see below).

12.4 HOMOLOGOUS RECOMBINATION AND REPAIR

The ideal method for correcting a defective globin gene and for solving the problem of low gene expression is to replace the abnormal DNA region with the normal sequence using homologous recombination (Smithies and Maeda, 1995). Accordingly, a mutated β globin gene has been corrected in cell lines, including embryonic stem cells (Shesely *et al.*, 1991). However, this method cannot yet be applied to haematopoietic stem cells due to the low frequency of recombination at the right location, and the need for clonal selection and multiplication of the very rare 'corrected' stem cells.

A site-specific repair of a point mutation, responsible for β thalassaemia, has been obtained by transfection of specific chimeric oligonucleotides into stem cells *ex vivo* (Cole-Strauss *et al.*, 1996).

12.5 INDIRECT GENE THERAPIES FOR β THALASSAEMIA

With the current unpredictable delay in development of a successful direct gene therapy, either by gene addition or by gene replacement, several indirect approaches are being investigated (Table 12.1).

12.5.1 CORRECTION OF ABNORMAL SPLICING

β Globin gene mutations resulting in an abnormal acceptor splicing site (eg. β IVS 1-110), which is much more potent than the normal intact site, are most frequent in Eastern Mediterranean countries, and are responsible for a severe β thalassaemia syndrome. It is possible in cultured cells to restore normal splicing to the 100% level and to recover a functional β globin mRNA by the use of antisense oligonucleotides masking this abnormal splicing site (Dominski and Kole, 1993). It should be possible to transfer a gene coding for the antisense RNA, transcribed under the control of globin promoter and LCR, into stem cells in order to express the antisense gene at the end of the erythroid differentiation.

12.5.2 ACTIVATION OF COMPENSATING GENES

Another indirect approach that can compensate for the defective β globin gene consists of activating a normally silent gene having the same or similar function. This is the case of the γ globin genes of Hb F which when expressed decrease the clinical severity of haemoglobin disorders (Stamatoyannopoulos and Nienhuis, 1994). The levels of Hb F increase upon hydroxyurea treatment, and lead to a decrease in the severity of sickle cell disease (Charache *et al.*, 1992, 1995). Other cytotoxic or chromatin modifying agents also stimulate the *in vivo* expression of the γ globin genes and, in addition, that of the erythropoietin gene (Rodgers *et al.*, 1993). Therefore, within the scope of gene therapy for haemoglobinopathies, different potential strategies should be considered for achieving increased expression of the γ globin genes. One such approach is the transfer of the erythropoietin (Epo) gene into cells so that they are able to secrete pharmacological doses of Epo *in vivo*.

12.5.3 ERYTHROPOIETIN GENE TRANSFER

The rationale of this approach stems from the beneficial effect of Epo seen in patients with sickle cell anaemia (Cozma *et al.*, 1995; El Hazmi *et al.*, 1995; Rodgers *et al.*, 1993) and β thalassaemia (Aker *et al.*, 1995; Olivieri *et al.*, 1995; Rachmilewitz *et al.*, 1995). However, effective doses are high, and therefore costly, precluding the routine use of recombinant Epo.

Animal models have been used to evaluate the effect of large doses of Epo on Hb F expression. In baboons, very high doses of Epo induced more than 60% of Hb F (Al-Khatti *et al.*, 1987). Recombinant Epo was able to compensate the anaemia of β thalassaemic mice. This was associated with a reduction of the reticulocyte count, reflecting an improvement of erythrocyte survival time, with little increase in the haematopoietic mass (De Franceschi *et al.*, 1996).

In β thalassaemic patients, activation of the γ globin gene (and β when expressed) must be high enough to improve the rate of erythropoiesis and to increase the red cell life-span. Such activation might also reduce the bone marrow expansion of poorly transfused patients with Cooley's anaemia.

An early study has shown that continuous expression of the Epo gene, following transduction of long-term repopulating stem cells with retroviral vector, was able to compensate the genetic defect in β thalassaemic mice (β major gene deletion in Hbb thal 1 mice) by activating the expression of the β minor gene (Villeval *et al.*, 1994). However, the expression level of the Epo transgene was not predictable, e.g. too high or too low, rarely at the right level and often transient, and the recipient β thalassaemic mice had to be irradiated before the transplantation of autologous and genetically modified bone marrow cells. Some degree of *in vivo* control of Epo transgene expression has been observed in retrovirally transduced myoblasts induced with doxycycline following transfer into syngeneic normal mice (Bohl, *et al.*, 1997).

The engraftment of neo-organs (Moullier *et al.*, 1995) formed from fibroblasts transduced with Epo cDNA gene under the control of the phosphoglycerate kinase (PGK) promoter has provided long-term expression (over 6 months) of the Epo transgene in normal mice. Levels of Epo and haematocrit depended on the number of genetically modified fibroblasts present in neo-organs (Naffakh *et al.*, 1995). However, compared with that in normal mice, the increase of haematocrit in β thalassaemic mice occurred for only a very short time. This may be partly due to the high concentrations of endogenous Epo in β thalassaemic mice, its substitution by transgenic Epo or for other, still unknown, reasons.

A high-titre adenoviral vector has also been evaluated for Epo transgene expression. The absence of integration, dilution of the Epo transgene with time and the immune response towards the cells expressing the adenoviral proteins were potential risks of low or transient expression. However, after a single intravenous dose of adenoviral Epo vector particles, an erythrocytaemia was induced for more than 6 months in normal mice (Descamps *et al.*, 1994). The long-term expression of an Epo transgene has also been obtained with an AAV vector by direct injection into muscles of normal mice (O. Danos, personal communication).

12.5.4 REDUCTION OF α GLOBIN GENE EXPRESSION

While β thalassaemia is due to a deficiency in β globin chain synthesis, it is well known that the clinical severity of the β thalassaemia depends on the imbalance between α and non- α globin chain synthesis and more specifically on the amount of free α haemoglobin chains (unbound to the β or γ chains), which are unstable and precipitate in erythrocytes and their precursors.

These unpaired α globin chains contain haem and iron, which oxidise and are catalysts forming highly toxic oxygen radicals, which induce haemolysis and ineffective erythropoiesis. An increase in the γ globin chains of Hb F or reduction of the α chains (e.g. due to co-inherited α thalassaemia determinants) reduces the globin chain disequilibrium and the clinical severity of β thalassaemia. In contrast, additional α globin genes (triplication) usually enhance the severity of the heterozygous state of β thalassaemia (Cao *et al.*, 1995). A reduction of 20–30% of the free α globin chains should transform a severe β thalassaemia syndrome into β thalassaemia intermedia, not requiring transfusion.

Strategies that aim to reduce the amount of free α chains can target different levels, from transcription to translation, of α globin gene expression. At the transcriptional level, α globin gene expression could be reduced with targeted inactivation of one of the two α genes, or of the HS-40 site of the α gene LCR, by using a homologous recombination methodology *ex vivo* (Bernet *et al.*, 1995). It should also be possible to use an 'antigene' coding for RNA forming a triple helix to inhibit a transactivating site (HS-40 or the α gene promoter). At the post-transcriptional level, an 'anti-RNA' gene coding for an antisense RNA (Ponnazhagan *et al.*, 1994) inhibited the processing of the α globin mRNA. It should also be possible to direct a ribozyme or a nuclease H in order to specifically digest the α globin mRNA or its precursors (Thompson *et al.*, 1995). The transcription of either antigene or anti-RNA gene should be under the globin gene regulatory elements in order to coordinate the expression of the α globin and anti α globin genes.

12.5.5 OTHER THERAPEUTIC GENES

Another approach, at the post-translational level, could consist of increasing the proteolysis of the α globin chains. Such a proteolytic system exists in the reticulocytes of β thalassaemic heterozygotes, asymptomatic in spite of 50% impaired α chains, because most of them are proteolysed. When the α globin chain concentration is above a certain threshold, as occurs in homozygous β thalassaemia, the α globin chain proteolytic process is probably saturated, excess α globin chains precipitate, and haemin and iron accumulate. It may be possible to improve the survival of erythrocytes and their precursors by the addition of a gene coding for a protease that specifically degrades free α globin chains without altering other cellular proteins.

The abnormal oxidation process, which is finally responsible for the cellular defects in β thalassaemia, could perhaps be reduced or prevented by the transfer of a gene coding for a protein able to scavenge the free radicals, or to bind the free iron or haemin released from unstable α globin chains that are responsible for oxidation and ineffective erythropoiesis.

12.6 SICKLE CELL DISEASE

Somatic gene therapy for sickle cell disease is more difficult to devise than that for β thalassaemia, because the β^s globin gene is expressed at high level and the gain of a new function, Hb S polymerisation, must be inhibited. In addition to several of the strategies described above, which are common to sickle cell disease and β thalassaemia, two specific strategies are under development: (i) the transfer of a gene coding for a globin with anti-sickling properties and (ii) the transfer of a gene coding for a bisphosphoglycerate mutase that degrades 2,3-bisphosphoglycerate (Garel *et al.*, 1994).

The first and direct approach consists of site-directed mutagenesis of codons 22 and 87 of the β globin gene to inhibit contacts in the polymer of haemoglobin as observed with Hb F and Hb A₂ (McCune *et al.*, 1994). This type of transgene has been shown to be stable and expressed in MEL cells transduced with retroviral vectors (Takekoshi *et al.*, 1995).

A new and very interesting approach to sickle cell disease is mutation repair by transfection of a chimeric, double-stranded, oligonucleotide targeting the codon 6 mutation in the β^s globin gene (Cole-Strauss *et al.*, 1996).

An indirect approach for specific anti-sickling gene therapy was deduced from the observation that a recombinant variant of 2,3-bisphosphoglycerate mutase having acid phosphatase homology has acquired a high activity of 2,3-bisphosphoglycerate phosphatase activity (Garel *et al.*, 1994). The transfer of the recombinant enzyme, in the red blood cells, decreased the 2,3-bisphosphoglycerate level *in vitro* and may be of interest for somatic gene therapy *in vivo*.

12.7 FUTURE PROSPECTS

Direct gene transfer using high titres of infectious viral vectors has produced high transduction efficiency of stable constructs and their expression in normal haematopoietic cells *in vitro* and in various human and mouse erythroid cell lines. This suggests the possibility of transduction and expression of globin genes *in vivo*. Studies of transduced human stem cells with long-term repopulating activity in immunodeficient animal models have become possible. Immunodeficient mice (Cashman *et al.*, 1997; Dick *et al.*, 1995) or foetal sheep grafted with CD34 + cells and stromal cells can be used to evaluate the transduction and expression of the transgene in all lineages derived from very primitive human stem cells *in vivo*. Using cytokines, it is now possible to stimulate the *ex vivo* multiplication of these primitive stem cells, and to avoid the chemoablation or whole body irradiation that is difficult to accept before grafting the transduced stem cells to patients with haemoglobinopathies.

A major problem, as yet unsolved, is to maintain a high level of expression of the β globin transgene *in vivo*. It has been demonstrated that retroviral sequences inhibit β globin gene expression in the fertilised eggs of mice after transfer of the provirus construct used for somatic gene therapy (McCune and Townes, 1994). Most of the studies of β globin gene transfer have been successful in culture, but not *in vivo*. One reason for this is that the cell lines used *in vitro* have a much lower endogenous globin gene expression than erythroblasts *in vivo*. Therefore, a 1:1 ratio of transgene and endogenous globin gene expression in MEL or K562 cell line is not readily achievable *in vivo* in the long term.

In the future, transfer of large DNA fragments, including the whole globin gene cluster, may be developed. This will require *ex vivo* multiplication and manipulation of stem cells. Cell targeting will probably have to be improved by specific binding of the vector to specific receptors. Fusogenic molecules may improve cytoplasmic transfer of DNA. AAV motifs may be designed to integrate the globin gene into the AAV-specific chromosomal site. All the present studies are converging to produce the ideal vector system using the high efficiency of transduction of viral vectors, but without the drawbacks of either retroviral or AAV vectors. The use of a human artificial chromosome may avoid random integration of the transgene in the cellular genome (Huxley, 1994).

The time when direct and efficient globin gene therapy will be achieved is difficult to foresee, therefore indirect gene therapies should be developed. They could be useful for the patient in reducing the burden of both the disease and current 'unpalatable' treatment regimes. These indirect therapies may reduce morbidity, mortality and the personal, familial, social and economic impacts of haemoglobinopathies, which remain severe and disabling genetic defects.

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13 Gene Therapy Approaches to Duchenne Muscular Dystrophy

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13.1 INTRODUCTION

Duchenne muscular dystrophy (DMD) is a severe neuromuscular disorder, characterised by progressive muscle wasting. Affected individuals are generally confined to a wheelchair before the age of 12 years and rarely survive into the third decade of life. DMD is an allelic X-linked recessive condition, being limited to males and transmitted by female carriers. The disease has a worldwide incidence affecting approximately 1 in 3500 live male births, with approximately one-third of the cases arising sporadically with no previous family history (Emery 1993). The disease stems from abnormalities in a genetic locus located on the short arm of the X chromosome, which codes for a multifunctional protein termed dystrophin. The high incidence rate and the severity of the disease make development of an effective therapy vital; gene therapy presents one of several approaches under investigation.

13.2 CLINICAL AND PATHOLOGICAL FEATURES

DMD is a well-defined homogeneous disorder, presenting a wide spectrum of clinical conditions. The effects of DMD on tissue musculature are consistent and always severe. Although the disease is present at birth, clinical signs are generally not manifested until walking is delayed or other mobility functions become obviously impaired. Increased lumbar lordosis and a distinct enlarging and firming of the calf muscles are indicative, associated with a general broad-based gait apparent from the ages of 3–6 years. Increasing difficulties in ambulation ultimately lead to wheelchair dependence by the age of 12 years (Emery 1993), where clinical conditions generally accelerate,

with contractures and scoliosis presenting major patient management problems (Miller and Hoffman 1994).

The skeletal musculature may be chronologically the first and ultimately the most severely affected tissue in DMD, but a number of other clinical conditions are associated with the cardiac and smooth musculature. Cardiac complications are frequent in DMD, being present in more than 80% of patients, but overt cardiac failure is a terminal event in only approximately 10% of DMD patients (Quinlivan and Dubowitz 1992). The majority of patients die of overt pulmonary failure early in the third decade of life, with forced vital capacity generally dropping to below 1 litre by the ages of 12–14 years (Bushby and Gardener-Medwin 1993). Smooth musculature has the least well-documented pathology and the clinical significance has been debated. Non-progressive cognitive impairment has been reported to manifest in approximately 50% of DMD patients (Bresolin *et al.* 1994). However, the symptoms are relatively mild overall and vary in expression, with verbal IQ and short-term memory reported to be mainly affected, with no significant statistical correlations between genetic data and psychometric assessment (Bresolin *et al.* 1994).

Becker muscular dystrophy (BMD) is a milder allelic form of DMD, manifesting a similar pattern of progressive proximal weakness to DMD, but with delayed presentation (Bushby and Gardener-Medwin 1993). The clinical phenotype of BMD is also much more heterogeneous than DMD in terms of age of onset, rate of disease progression, distribution and severity (Vainzof *et al.* 1993). The effects on ambulation are generally less severe in BMD, with some patients never losing the ability to walk (Emery 1993).

13.3 THE DYSTROPHIN GENE AND ITS PRODUCTS

Since the early 1980s remarkable progress has expanded our knowledge of the pathogenesis and function of this disease, initiated by the precise localisation of the DMD locus to the short arm of the Xp21 chromosome (Davies *et al.* 1983). Elucidated as the largest known gene to date, it spans at least 2400 kb, consisting of 79 exons with 99.4% of the gene consisting of introns. This immense gene codes for a 14 kb mRNA transcript comprising elaborate transcriptional and splicing controls, translating to a 427 kDa protein, termed dystrophin (Brown and Dickson 1994).

The amino acid sequence of dystrophin indicates four distinct structural domains (Figure 13.1). The N-terminal domain, comprising the first 240 amino acids, displays high sequence homology to the actin-binding domains of α -actinin and β -spectrin and has been shown to bind to specific sequences of F-actin, possibly in a calcium-dependent regulated manner (Winder and Kendrick-Jones 1995). The central rod domain consists of 24 homologous

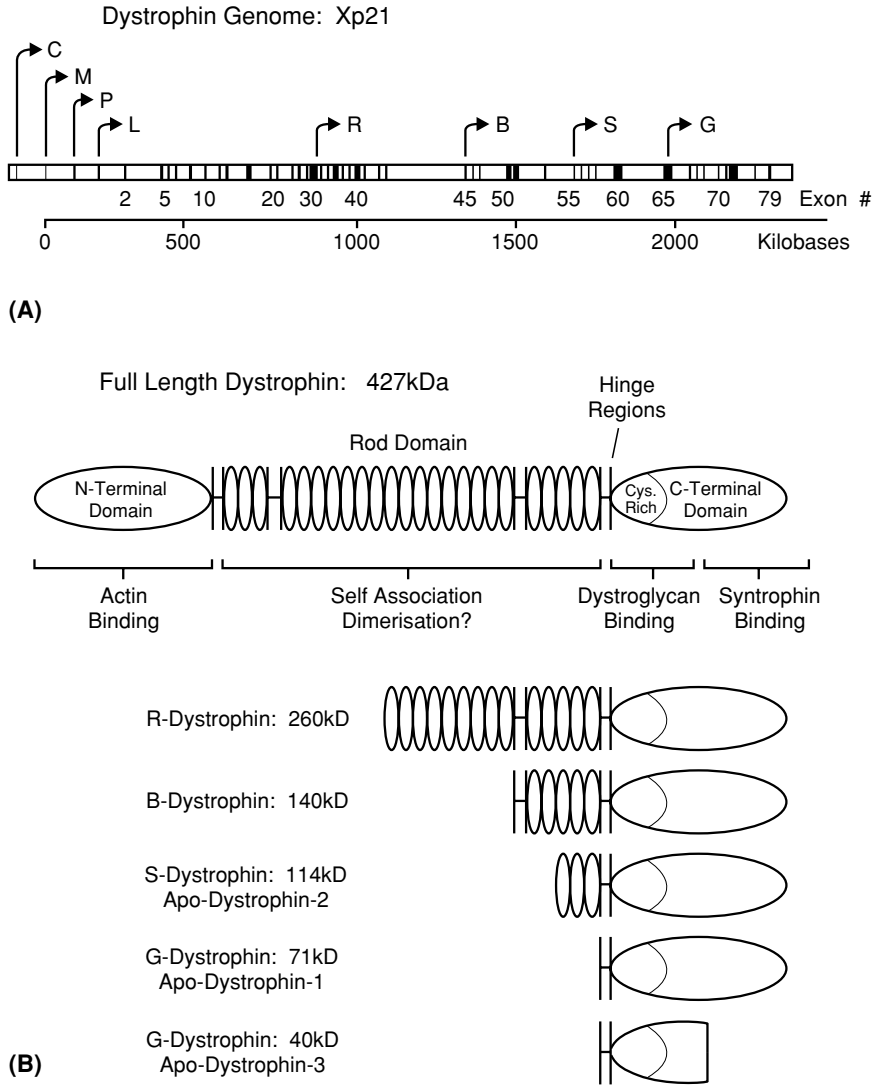


Figure 13.1. (A) Schematic representation of the dystrophin gene showing the approximate locations of the 79 exons and the eight presently identified promoters. (B) Proposed domain organisation of the main dystrophin gene product and the predicted domain organisation of the smaller protein transcripts; the *N*-terminal domains of the truncated proteins contain short unique sequences not present in the *N*-terminal of the full-length transcript.

repeats, averaging 109 amino acids, also with high homology to α -actinin and β -spectrin. The structure is predicted to promote α -helical coiled-coil interactions, adopting a rod-like structure 100–125 nm long, which could facilitate self-association (Blake *et al.* 1995). Hinge-like flexibility is believed to be conferred on the molecule, by the presence of four proline-rich regions, which are dispersed along the length of the rod (Blake *et al.* 1994). A cysteine-rich domain spanning residues 3080–3360 encompasses two calcium binding motifs present in calmodulin as well as α -actinin and β -spectrin, although its ability actually to bind calcium is debated (Ahn and Kunkel 1993). Finally, the C-terminal domain of 420 amino acids is believed to be specific to the dystrophin superfamily, presently consisting of dystrophin and a growing number of dystrophin-related proteins (DRP), including utrophin (DRP1), DRP2, Torpedo 87K protein (Roberts *et al.* 1996) and the dystrobrevins (Blake *et al.* 1996).

To date eight independent promoters have been elucidated as controlling the transcription from the DMD locus in cell-specific and developmentally controlled manners (Matsuo 1996). Four of these promoters initiate the transcription of full-length mRNA transcripts, differing by at least the first exon, while encoding the 427 kDa M (muscle), L (lymphoblastoid), C (cortical) and P (Purkinje) dystrophin proteins, containing all four structural domains. Additional S (Schwann) and G (glial) promoters control the expression of short C-terminal apo-dystrophin proteins. The S promoter transcribes a 5.6 kb mRNA transcript encoding a 116 kDa protein; termed Dp116 or apo-dystrophin-2, which is uniquely expressed in adult peripheral nerve (Blake *et al.* 1995). The G promoter transcribes at least two mRNA transcripts of 4.8 and 2.2 kb, termed apo-dystrophin-1 and apo-dystrophin-3, respectively, which have identical expression patterns, being abundant in brain and non-muscle tissues but undetectable in fully differentiated skeletal muscle (Tinsley *et al.* 1994). Recently two additional dystrophin transcripts of 260 kDa (Pillers *et al.* 1993) and 140 kDa (Lidov *et al.* 1995) were elucidated, initiated from novel R (retinal) and B (brain, CNS) promoters, respectively. However, the complexity of the dystrophin gene's transcriptional control and mRNA splicing being far greater than those of any other known cytoskeletal protein, it is probable that other small transcripts exist.

Both DMD and BMD are now known to be caused by mutations in the gene that codes for dystrophin. Mutation rates have been calculated as approximately $70\text{--}100 \times 10^{-6}$ genes per generation, which is much higher than for any other genetic disorder (Emery 1993). The exceptionally high incidence and the vast range of mutations associated with the DMD genetic locus are probably reflections of the huge scale of the gene, which provides a large target for mutagenic agents. However, a number of hotspots in the dystrophin gene account for a large proportion of the mutations which exist, indicating specific targets for mutagenesis (Brown and Dickson 1994). In the

majority of cases the clinical severity of the phenotype can be correlated to the nature of the molecular deletion (Monaco *et al.* 1988). DMD phenotypes generally arise from mutations that disrupt the genomic reading frame, resulting in non-functional gene products, and hence the phenotype is always homogeneously severe due to a functional deficiency. BMD phenotypes, however, generally arise from mutations/deletions which maintain the reading frame, producing abnormal protein products that are altered but that maintain some functionality, with varied phenotypes that are mutation dependent (Bushby 1992).

13.4 THE LOCALISATION AND FUNCTION OF DYSTROPHIN

Dystrophin has been demonstrated to be a major component of the cytoskeleton of all normal muscle fibres, being accurately located to the subsarcolemmal membrane and present in full levels soon after muscles form during foetal development (Arahata *et al.* 1988). Early in myogenesis normal undifferentiated myoblasts contain undetectable levels of dystrophin expression. The appearance of dystrophin coincides with the spontaneous fusion of these mononuclear myogenic cells to form post-mitotic, multinucleated syncytia (Miranda *et al.* 1988). However, in dystrophic muscle, dystrophin is absent or markedly deficient, resulting in marked variations in fibre size, with scattered fibres undergoing regeneration, degeneration or necrosis (Miranda *et al.* 1988). The absence of dystrophin does not result in immediate cell death of all muscle fibres, with most myofibres in DMD and BMD remaining functional early in the disease process. However, regeneration of damaged muscle fibres progressively fails to compensate for increasing degrees of muscle fibre degeneration, being further hampered by the gradual deposition of fibrotic connective tissue at severely affected sites, resulting in increasing instability of myofibres (Miller and Hoffman 1994).

Although dystrophin has been reported to show comparable expression in differentiated skeletal, cardiac and smooth muscles (Chevron *et al.* 1994) the absence of dystrophin has significantly different clinical and phenotypical effects in each tissue. The effect of dystrophin deficiency on a particular tissue has been predicted to be related to the degree of axial stress exerted upon the myocytes (Cziner and Levin 1993). In considering the morphological and physiological characteristics of the muscles, skeletal myocytes are multinucleated and linear, with forces being generated exclusively along the major axis. Cardiac myocytes, however, are mononuclear and branched, with forces being more radially generated and hence less uniformly directed along the muscle, which is therefore less severely affected than skeletal muscle. The spindle shape of smooth muscle, with even more random

association and very extensive connective tissue networks, has its forces even more widely distributed and hence clinically is the least affected tissue. This hypothesis clearly assigns a function for dystrophin, physically reinforcing the sarcolemma against the axial forces exerted upon the myocyte and providing membrane stability during repeated cycles of contraction (Pasternak *et al.* 1995).

Electron microscopy studies have established dystrophin in a sub-sarcolemmal location tightly associated in an oligomeric complex with a number of dystrophin-associated proteins (DAPs) and glycoproteins (DAGs), termed the dystrophin-associated glycoprotein complex (DAGC) (Figure 13.2). The cysteine-rich domain and first half of the carboxyl terminus is believed to anchor dystrophin to the sub-sarcolemmal face of the muscle membrane primarily through interactions with the transmembrane glycoprotein, β -dystroglycan. This 43 kDa DAG is central to the complex and provides a link to the extracellular matrix through binding to α -dystroglycan, a 156 kDa extracellular DAG which binds to merosin, the muscle isoform of laminin (Tinsley *et al.* 1994). Further associated with β -dystroglycan are at least five transmembrane DAGs, which form the sarcoglycan complex, termed α , β , γ and δ sarcoglycan, and an unclassified 25 kD DAP, together with other undetermined proteins (Beckman 1996). Finally, a cytoplasmic complex exists in which dystrophin is associated via its carboxy terminal's latter half to α - (muscle), β 1- (ubiquitous) or β 2- (neuromuscular junctions NMJ) syntrophins (59 kDa DAPs) (Yang *et al.* 1995a), as well as to a 94 kDa DAP, A₀ (Tinsley *et al.* 1994), and possibly other cytoplasmic signalling molecules, including nitric oxide synthase (NOS) (Brennan *et al.* 1995).

Considering the N-terminal linkage of dystrophin to the F-actin components of the intracellular matrix, the entire membrane cytoskeleton may provide a linkage between contractile proteins in the intracellular myofibrillar matrix and the extracellular connective tissue of the basal lamina (Tinsley *et al.* 1994). Hence, the absence of any of the links in the chain of interaction from the actin cytoskeleton, through dystrophin and the DAGC to the extracellular matrix, could compromise the integrity and flexibility of the sarcolemma. The reality of this function is emphasised in that an autosomal recessive muscular dystrophy with a DMD phenotype has been associated with a primary deficiency of most of the components of the DAGC (Table 13.1).

Myofibre leakiness appears to be a primary consequence of the lack of dystrophin, with elevated intracellular calcium accumulations in DMD myofibres present from birth. High intracellular calcium levels are harbingers of cell necrosis, and the breakdown of sarcolemma precedes muscle cell necrosis in DMD (Fong *et al.* 1990). In normal exercising muscle, transient breaches of the myofibre membrane are believed to induce elevated serum creatine kinase (SCK) and subsequent muscular hypertrophy as a natural wound

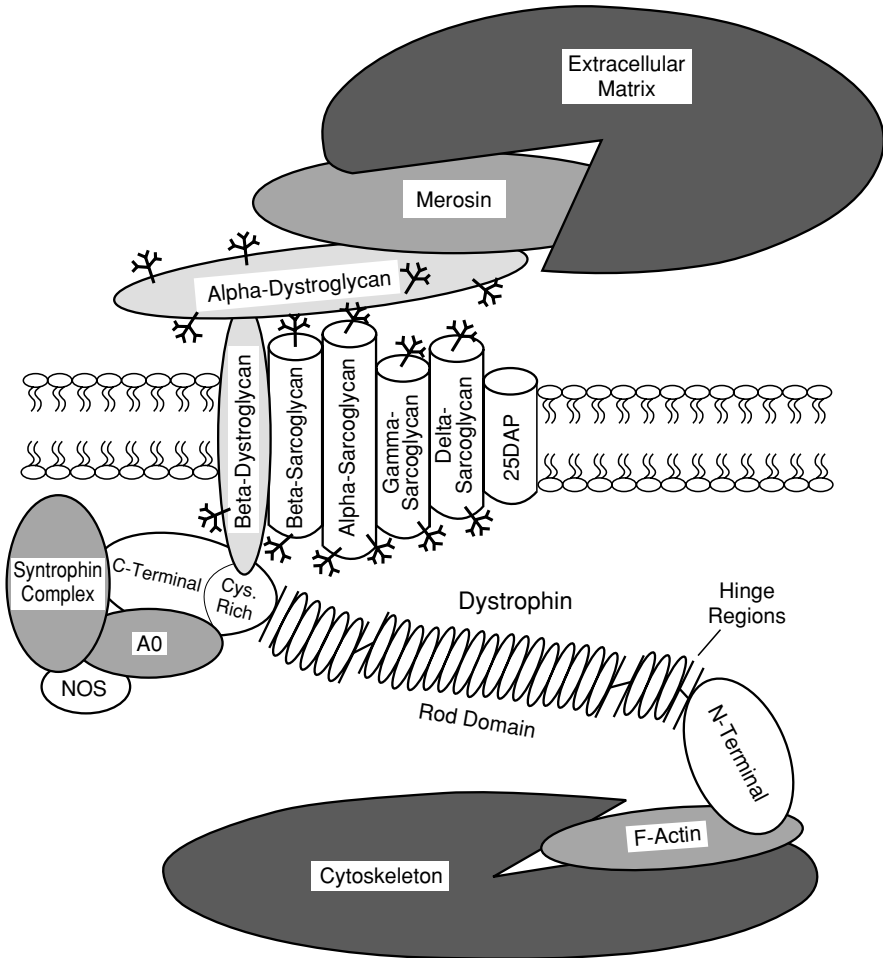


Figure 13.2. Schematic representation of the dystrophin associated glycoprotein complex (DAGC) localised at the sarcolemmal membrane of all normal muscle cells, emphasising the structural link between the intracellular cytoskeleton and the extracellular matrix.

repair mechanism. However, in dystrophic muscle, transient breaches are the consequence of a biochemical defect, which might stimulate the effect of exercise at the cellular level, erroneously signalling myofibre hypertrophy and leading to endomysial fibrosis and subsequent muscle wasting (Gaschen *et al.* 1992).

Dystrophin shows a lower expression in brain tissues than in muscle, with a high abundance in cerebellum, hippocampus and cerebral neocortex, and

Table 13.1 Components of the dystrophin associated glycoprotein complex (DAGC)

Location	DAGC component	Size (kDa)	Genetic locus	Synonyms	Associated diseases ^a
Extracellular Trans-membrane	α -Dystroglycan	156	3p21	156DAG	—
	β -Dystroglycan	43	3p21	A3a, 43DAG	—
	α -Sarcoglycan	50	17q	A2, 50DAG, Adhalin	LGMD2D
	β -Sarcoglycan	43	4q	A3b, 43DAG	LGMD2E
	γ -Sarcoglycan	35	13q	A4, 35DAG	LGMD2C
	δ -Sarcoglycan	35	5q	—	LGMD2F
	25DAP	25	—	A5	—
Cytoplasmic	Dystrophin	427	Xp21	—	DMD/BMD
	α -Syntrophin	59	20q11	α -A1	—
	β_1 -Syntrophin	59	16	β_1 -A1	—
	β_2 -Syntrophin	59	8q23–24	β_2 -A1	—
	A0	94	—	—	—
Extracellular matrix	Merosin	400	6q22–23	Laminin- α 2-chain	CMD

^aDiseases associated with the absence of specific components of the complex: LGMD, limb-girdle muscular dystrophies; CMD, congenital muscular dystrophy.

Sources: Beckman and Bushby 1996; Tinsley *et al.* 1944).

barely detectable levels in the basal ganglia and brain stem. As the hippocampus and cerebral neocortex directly subserve emotions, memory and cognitive processes, it is highly likely that dystrophin is directly involved in DMD-associated mental retardation (Bresolin *et al.* 1994). Considering the abundance of expression of the smaller C-terminal apo-dystrophin proteins in brain tissue, they may have functions in cognitive processes quite diverse to the function of full-length dystrophin in muscle (Blake *et al.* 1994). However, although Bresolin *et al.* 1994 demonstrated cerebral hypometabolism in all DMD patients examined, whether cognitive impairment in DMD is a primary or secondary effect has yet to be determined.

13.5 MODEL SYSTEMS OF DMD

Biochemical and genetic homologues of DMD exist in mice, dogs and cats that lack dystrophin expression, with skeletal muscle fibres observed to undergo spontaneous necrosis and degeneration. The well-established murine model, the mdx mouse (Bulfield *et al.* 1984), contains a non-sense point mutation in the dystrophin gene (Sicinski *et al.* 1989). The disease phenotype is characterised by a sudden onset of florid myofibre necrosis occurring 2–3 weeks post-birth, with lesions resembling those observed early

in DMD pathology. However, despite an early phase of slight muscular weakness, the animals are not clinically compromised until beyond 18 months, and, in contrast to human DMD, the muscles proceed to grow larger and stronger than normal and appear to remain healthy and active throughout a somewhat normal life span (Coulton *et al.* 1988). This has been attributed to effective muscle regeneration, which more than compensates for the degenerative state, as well as a lack of fibrosis and a distinct attenuation of myonecrosis in older mice (Carnwath and Shotton 1987). However, the mdx mouse diaphragm, as well as other muscles, has been shown to exhibit patterns of degeneration, fibrosis and severe functional deficit comparable to DMD limb muscle, which has greatly aided therapeutic testing (Stedman *et al.* 1991).

Feline X-linked dystrophy bears more resemblance to mdx than DMD (Gaschen *et al.* 1992), whereas canine X-linked muscular dystrophy (CXMD or xmd dog) involves muscle fibre necrosis and regeneration, with marked endomysial and perimysial fibrosis almost identical to those which occur in DMD patients (Valentine and Cooper 1991). However, the relatively long generation time in the canine model and the scale of animal care can prove slow and expensive in experimental terms, hence most studies have focused on the mdx mouse, which has undoubtedly accelerated our knowledge and understanding of DMD.

13.6 THERAPEUTIC APPROACHES TOWARDS THE TREATMENT OF DMD

The high incidence of DMD, the hardship the disease imposes and the potential transmission by healthy female carriers makes detection of healthy heterozygous carriers one of the major goals in the prevention of the disease. With only approximately 5–10% of carriers manifesting some degree of muscle weakness, due to individual variations in random inactivation of the X chromosome (Bonilla *et al.* 1988), the single most reliable test for healthy carriers remains the SCK level, provided due care and attention is given to the various technical and biological factors that can affect it, including age and pregnancy. However, new direct methods are being developed which focus on identifying the mutation itself (Bonilla *et al.* 1988).

In recent years slight decreases in the incidence of DMD have been observed, generally due to genetic counselling, carrier detection tests and prenatal foetal sexing (Emery 1993). However, because one-third of cases result from spontaneous mutations, efficient therapies to complement the acute diagnostic technology now available are essential.

The availability of cDNAs encoding recombinant dystrophin peptides has allowed the possibility of somatic gene transfer as a therapeutic approach to

treat dystrophin deficiencies (Dickson *et al.* 1991). In establishing the physiological effect of restoring dystrophin gene expression and its effect on the link between the extracellular and intracellular matrices, transgenic mice have been invaluable. The complete correction of the morphological and immunohistochemical symptoms of muscular dystrophy has been reported in transgenic mdx mice expressing the full-length dystrophin cDNA, accompanied by the restoration of the DAGC and subsequent restoration of normal muscle function (Wells *et al.* 1995). Additionally, the overexpression of dystrophin in skeletal or cardiac muscle was not associated with any deleterious side effects, indicating that tight control over dystrophin expression may not be necessary (Cox *et al.* 1993). The restoration of normal muscle function upon gene replacement with these recombinant dystrophin cDNAs greatly encourages an effective therapy to cure DMD.

At present the size of the full-length dystrophin cDNA exceeds the packaging limits of most viral gene delivery systems. To circumvent this limitation current research has utilised alternative recombinant dystrophin cDNAs, in particular a 229 kDa (6.3 kb) dystrophin cDNA isolated from a BMD patient with an extremely mild phenotype (England *et al.* 1990). This mini-dystrophin locates to a correct sarcolemmal location and remains at least partially functional despite a large in-frame deletion in the central rod domain, extending from exon 17 to exon 48. Transgenic mdx mice harbouring this 6.3 kb mini-dystrophin cassette exhibit a distinct phenotype reversal, suggesting that this mutated recombinant dystrophin is highly functional (Wells *et al.* 1995).

Considering the wide pathology of DMD, the level of dystrophin expression theoretically required to normalise dystrophic muscle is an extremely complex question. Expression of 20% of endogenous levels of the 427 kDa protein appear to be protective in mdx diaphragm, whereas higher levels of expression are probably required in quadriceps muscles (Phelps *et al.* 1995). In comparison, with the mini-dystrophin protein > 30% endogenous diaphragm expression would appear to be therapeutic (Wells *et al.* 1995), indicating that although a restoration of the DAGC is also observed with the 229 kDa mini-dystrophin, a less stable membrane association is predicted, therefore higher expression levels will probably be required to achieve the same therapeutic effects as with the 427 kDa dystrophin (Wells *et al.* 1995).

13.7 CELL THERAPY FOR DMD: MYOBLAST TRANSFER

Transplantation of healthy donor myoblasts into dystrophic tissue is an extensively researched therapeutic option, initiated by the finding that injected muscle precursor cells (mpc) of one phenotype can fuse with muscle fibres of a different genotype and express donor genes (Watt *et al.* 1984). The

injection of healthy human or mouse mpc into mdx dystrophic muscle has now been well documented to induce the expression of substantial amounts of normally sized dystrophin with the correct localisation (Partridge *et al.* 1989; Vilquin *et al.* 1995). Although a patchy profile of dystrophin immunostaining is observed, there is evidence of increased fibre survival (Morgan *et al.* 1993).

The promising results in mdx mice rapidly encouraged human trials. However, to date the results have been disappointing, with the overall efficiency being extremely low, and no real long-term clinical improvements observed (Mendel *et al.* 1995). The inefficiencies of this technique have been ascribed to a number of factors that are difficult to circumvent outside the realms of an animal model. The failure of mpc to fuse with non-regenerating muscle fibres greatly limits the therapy, and the fact that few mpc are observed to incorporate into growing muscle tissue confounds the need for therapy early in life, due to the progressive nature of the disease (Partridge *et al.* 1989). The number and viability of injected myoblasts, as well as muscle pretreatment and the injection protocol itself, have proved to be important factors, but the immune system plays a dominant role (Vilquin *et al.* 1995). Even in hosts that are major histocompatibility complex- (MHC-) compatible and tolerant, the lysis of myotubes by alloreactive cytotoxic T cells and natural killer (NK) cells has been observed within the first few days, despite immunosuppression (Roy *et al.* 1993). Circulating antibodies directed against epitopes of donor cells as well as to dystrophin itself have also been reported, but dystrophin incompatibility alone is not sufficient to induce immunological rejection (Vilquin *et al.* 1995).

The transplantation of patient-derived, autologous myogenic cells that have been transduced with recombinant dystrophin and then reintroduced has an advantage over heterologous transplantation in circumventing immune rejection. However, as the proliferative potential of satellite cells derived from dystrophic muscle has already been exhausted due to the repeated cycles of degeneration and regeneration a major obstacle arises in obtaining sufficient quantities of functional cells to inject. Also, although injected mpc have been reported to show some migratory properties (Morgan *et al.* 1993), the scale is very limited, with the vast muscle pathology and the inaccessibility of many muscles, including the heart and diaphragm, making myoblast transfer relatively impractical.

13.8 GENE THERAPIES FOR DMD

Currently gene therapy is aimed at the level of gene augmentation, where a normal gene is introduced into cells such that it can function to produce sufficient quantities of the correct gene product to compensate for the lack of

expression of the mutant host gene. A number of genetic complementation strategies are being developed aiming to deliver copies of a recombinant dystrophin gene construct into muscle cells both *ex vivo* and *in vivo*. The ultimate goal is long-term, sufficiently regulated expression of the transferred gene, achieved by a single, lifetime treatment involving a simple, non-invasive, safe and efficient gene delivery which can be incorporated into clinical practice.

13.8.1 DIRECT DNA INJECTION

In terms of gene therapy the simplest, least expensive and possibly the safest procedure is the direct introduction of pure closed circular DNA or RNA into a desired tissue. Direct intramuscular injection of plasmid DNA into mouse skeletal muscle *in vivo* has been demonstrated to result in stable gene transfer. The injected DNA locates to the nucleus extrachromosomally in a circular form, with the post-mitotic nature and longevity of muscle fibres permitting the stable expression of transferred genes even in extrachromosomal states (Wolff *et al.* 1992).

However, direct DNA transfer has been reported to result in highly variable reporter gene expression, due predominantly to the highly variable distribution of the injected substance. Preinjections of relatively large volumes of hypertonic (25%) sucrose solutions have been demonstrated to reduce this variability due to hydrostatic pressures forcing fibres apart, increasing the sample distribution as well as the mean level of expression (Davis *et al.* 1993a). However, injections of such large volumes, besides being painful to the subject, result in significant fibre damage, which should ideally be kept to a minimum (Davis *et al.* 1993a). Higher transfection efficiencies have also been observed in regenerating muscle induced experimentally by pretreatment with the mytotoxic local anaesthetic, bupivacaine (BPVC) (Wells 1993). This may be advantageous towards gene therapy for DMD as regenerative fibres are numerous in the early pathology of the disease.

Muscle tissue is particularly suited to DNA uptake and expression, being considerably higher in striated muscle than any other tissue (Acsadi *et al.* 1991). The uptake efficiency in mice has been demonstrated to be dependent on the age and sex of the recipient (Wells and Goldspink 1992), with mononuclear muscle cell precursors appearing refractory to uptake, and only becoming efficient at certain levels of maturity (Davis *et al.* 1993b). Endomysial injection to mouse diaphragm has been reported to be safe and result in effective gene transfer with minimal myofibre damage at levels comparable to that in skeletal muscle pretreated with hypertonic sucrose or with induced regeneration. This is most probably due to the endomysium being less dense than skeletal muscle, favouring uptake due to better diffusion (Davis *et al.* 1993b). As the diaphragm of the mdx mouse has been

reported to be the tissue which most reflects the pathology in DMD (Stedman *et al.* 1991), this observation may aid the clinical evaluation of direct injection to treat dystrophin deficiencies.

Towards gene therapy for muscular dystrophy several groups have reported the stable expression of Becker-like and full-length dystrophins in mdx muscle after direct intramuscular injections of expression plasmids. The proteins have been reported to be correctly localised, targeting approximately 1% of fibres (Acsadi *et al.* 1991), and appeared to function to protect mdx fibres from degeneration (Danko *et al.* 1993). However, the low efficiencies and poor reproducibility of results, together with the prospect of treating the immense scale of affected muscle tissues in DMD particularly, constrain this technique. A number of mechanisms to enhance DNA uptake have, however, been proposed, including the complexing of DNA with a suitable medium to facilitate uptake, such as polycations (Kawai and Nishizawa 1984) or receptor ligands (Wagner *et al.* 1992; Wu *et al.* 1989), but recently most interest has focused on the use of cationic liposomes as vehicles for the transfer of recombinant genes into a variety of tissues both *in vitro* and *in vivo* (Alton *et al.* 1993), with efficiencies of up to 10% dystrophin cDNA transfer reported in primary cultures using liposome vehicles (Trivedi and Dickson 1995). However, a very recent study demonstrated dystrophin plasmid injection into the diaphragm, resulting in 15–20% efficiency and a significantly improved physiology (Decrouy *et al.* 1997), suggesting future scope for direct intramuscular gene transfer.

13.8.2 RETROVIRUS VECTORS

In terms of somatic gene transfer, replication-defective retroviruses offer a number of advantages over current delivery techniques. As well as providing significantly elevated targeting efficiencies and encoding no cytotoxic or immunogenic viral antigens, they result in efficient and stable integration of the transgene into the host chromosome, enabling a permanency that facilitates long-term expression (Miller *et al.* 1988). Retroviruses have been shown to infect muscle cells *in vitro* and initiate transgene expression (Smith *et al.* 1990). However, a number of fundamental obstacles limit the effectiveness of retrovirus gene transfer for DMD, including their inability to infect post-mitotic tissues such as muscle, due to their mitosis-dependent integration (Miller *et al.* 1990). Size limitations on DNA inserts of 9–10 kb further hamper their applicability, with research to date focusing on the 6.3 kb functional Becker-like mini-dystrophin cDNA. Recombinant retroviruses containing this minigene in place of the retroviral structural genes have been shown to be packaged into infectious viral particles and have been used to infect primary cultures of mdx myoblasts, resulting in the expression of the mini-dystrophin protein with the correct sarcolemmal location (Dunckley *et al.* 1992).

Direct *in vivo* intramuscular injections of this construct into mdx mice at 7–8 weeks postnatal life resulted in stable expression of mini-dystrophin with the correct sarcolemmal location in an average of 6% of myofibres for up to 9 months, together with the reappearance of the 43 kDa DAG, α -dystroglycan (Dunckley *et al.* 1993). Increased mitotic activity of regenerating dystrophic muscle is believed to enhance its suitability for direct retroviral-mediated transfer *in vivo*, with slightly improved results, up to 8% reported in experimentally induced regenerating mdx muscle (Dunckley *et al.* 1993).

In adult skeletal muscle, mitotic activity is restricted to satellite cells that can be targeted by recombinant retroviruses during regeneration. However, the relatively low-efficiency transduction most probably reflects the limited temporal availability of dividing satellite cells, as well as interference by the immune system greatly reducing the already short half-life of retroviruses *in vivo* (Fassati *et al.* 1995). Murine retroviruses have been shown to be sensitive to human complement-mediated lysis (Welsh *et al.* 1975). Current research is attempting to circumvent this by the modification of envelope glycoproteins to produce complement resistant pseudotype retroviruses (Takeuchi *et al.* 1994) or alternatively by the more drastic approach in the use of complement inhibitory drugs (Rother *et al.* 1995).

Recombinant retroviruses are now being constructed with internal promoter elements driving the transgene expression, avoiding restrictions associated with the endogenous long terminal repeat (LTR) promoter (Jahner *et al.* 1982). The use of muscle-specific regulatory regions to restrict their expression to differentiated muscle fibres enables the additional control of tissue specificity (Ferrari *et al.* 1995). In addition, the engineering of heterologous protein domains into the envelope glycoproteins, modifying their tropism, introduces the possibility of precise targeting of recombinant retroviruses to specific cell types (Kasahara *et al.* 1994; Somia *et al.* 1995).

An *ex vivo* approach of retroviral-mediated gene transfer into primary cultures of patient-origin myoblasts *in vitro* and autologous grafting back into the patient has produced some success (Salvatori *et al.* 1993), although this technique presents the same limitations as myoblast transfer. An alternative approach of direct injection of mitotically inactivated retroviral producer cell lines into regenerating skeletal muscle has resulted in much increased efficiencies of transduction and infection of satellite cells, which contribute to new fibre formation in the long term (Fassati *et al.* 1996).

Many refinements of *in vivo* strategies are required before clinical trials of retrovirus-mediated gene therapy to skeletal muscle can be attempted. Additionally, considering that cardiomyopathy is a major cause of death in DMD patients, as cardiocytes cannot regenerate they are expected to be refractory to retroviral-mediated gene therapy.

13.8.3 ADENOVIRUS VECTORS

Adenovirus vectors are attractive candidates for gene transfer to skeletal muscles due to their independence of host cell replication and low pathogenicity in humans, which together with their broad host range and high titres make widespread clinical treatment feasible. The major obstacle with current adenovirus vectors is the relatively tight constraints on the size of DNA packagable into virions, of up to approximately 105% of the wild-type genome (Bett *et al.* 1993). The capacity for foreign DNA insert is restricted to about 7.8 kb (Bett *et al.* 1994) and hence cannot accommodate full-length dystrophin cDNA, with current research focusing on the functional 6.3 kb Becker-like mini-dystrophin cDNA (England *et al.* 1990).

Recombinant E1/E3-deleted adenovirus vectors containing this 6.3 kb Becker-like dystrophin cDNA driven by constitutive RSV and CMV promoters have been demonstrated to deliver mini-dystrophin in culture (Dickson *et al.* unpublished; Ragot *et al.* 1993). *In vivo* studies involving single intramuscular injections of this adenovirus construct into neonatal mdx mice have shown efficient synthesis of significant amounts of the mini-dystrophin with the correct sarcolemmal localisation in 5–50% of fibres. This expression was reported to be apparent six months after the single injection, with no evidence of histopathological alterations due to cytotoxic immune responses (Vincent *et al.* 1993). The therapeutic significance of this delivery was reflected in significant functional protection of the dystrophic muscle to mechanical stress (Deconinck *et al.* 1996). Recombinant adenoviruses expressing reporter genes under the control of muscle-specific regulatory sequences have been shown to efficiently direct tissue-specific expression (Quantin *et al.* 1992). This important level of control has since been incorporated into mini-dystrophin viruses directing exclusive expression in myofibres (Alameddine *et al.* 1994).

The route of administration is a major factor in terms of transduction efficiencies to different tissues. Following systemic administration of recombinant adenovirus, a large proportion of cells are generally found to be infected and to express the transferred gene (Kass-Eisler *et al.* 1994). In terms of adenoviral-mediated gene therapy for muscular dystrophy, large numbers of dystrophin-positive fibres are observed in the heart, diaphragm and intercostal muscles, but not in limb muscles (Acsadi *et al.* 1996). Hence complex targeting strategies are likely to be required, including local administration to selected muscle groups and intra-arterial or intrapleural administration for heart, diaphragm and respiratory muscles. Additionally, the infectivity of muscle tissues by adenovirus vectors is very much dependent on the maturity of the tissue. Even at high titres mature muscle fibres, as compared to immature muscle, are not efficiently transduced (Acsadi *et al.* 1994; Dickson *et al.* unpublished). This may correlate with a higher surface density of

the available adenovirus internalisation receptor in immature muscle cells (Acsadi *et al.* 1994); however, immunological factors most probably play a dominant role. Significantly increased efficiencies have been observed in adult mice if abundant muscle regeneration is induced (Dickson *et al.* unpublished). However, in contrast to skeletal muscle, the transduction efficiency of cardiac muscle of adult mice is very similar to that observed in neonatal mice (Acsadi *et al.* 1995).

The longevity of adenovirus-directed foreign gene expression is seldom detected for periods longer than one year. Antigen-specific lymphocytic immune responses to virus infected cells, involving CD4+ and CD8+ T cell infiltrations, are believed to be responsible for both diminished transgene expression and part of the observed inflammatory responses. However, T cell responses are not predicted to be solely responsible, and minor non-antigen-specific destruction via NK cells or macrophages may also be involved (Yang *et al.* 1995). Additionally, humoral mechanisms of neutralising antibody production are believed to underlie an obstacle to repeated administrations (Yang *et al.* 1995b). Significant reductions in gene expression have been observed upon repeated adenoviral dosage which correlate inversely with neutralising antibody titre (Yei *et al.* 1994). However, it is predicted that repeated administration of low doses may be tolerated, albeit at decreasing levels of gene transfer (Bout *et al.* 1994).

Despite being defective of early region function (E1a/b), first-generation adenovirus vectors are prone to leaky transcription/translation of highly immunogenic late viral proteins, which initiate CTL responses and ultimately clearance of transgene expression (Yang *et al.* 1995b). Second-generation adenoviral vectors have focused on evading the immune interference by deleting (Gorziglia *et al.* 1996) or utilising temperature-sensitive mutations in the E2a gene (Yang *et al.* 1994), which ablate major late promoter activation by re-incorporating adenoviral immunosuppressive genes, e.g. gp19 from the E3 region (Poller *et al.* 1996).

Although the Becker-like mini-dystrophin protein retains some functionality, its transfer would still only impart a Becker-like phenotype to the recipient. Current research is focusing on the development of minimal sequence adenoviral vectors, in which all the structural genes are deleted, leaving only the sequences essential to direct genomic replication and packaging. Such vectors offer superior cloning capacities of inserts up to 37 kb, and because no viral structural genes are present, secondary immune responses are likely to be avoided. Pseudoadenoviral constructs containing the full-length dystrophin cDNA cassette (Dickson *et al.* 1991) have now been developed which can be assembled into infective adenoviral capsids by the use of so-called helper virus that supplies the structural genes *in trans* and has been demonstrated to successfully direct the expression of full-length dystrophin *in vitro* and *in vivo* (Haecker *et al.* 1996; Murphy *et al.* unpublished). However,

immune responses to UV-inactivated viruses have been shown to be fully competent, suggesting that primary helper T cell and B cell responses are to input viral proteins (Yang *et al.* 1995b). Hence, improvements in vector design that minimise viral protein expression will not address the problem of re-administration. Transient blunting or manipulation of the host immune response to the virus may be necessary at each administration (Lochmuller *et al.* 1996).

13.9 ALTERNATIVE THERAPEUTIC STRATEGIES

Recently a number of novel approaches to the therapeutic restoration/prevention of the dystrophic phenotype have been investigated. Antisense oligodeoxynucleotides (ODNs) are widely-used inhibitors of gene expression, offering the possibility of blocking expression of specific genes without changing the expression of others, involving targeted complement DNA association and sterically hindering the association of the genetic expression machinery. Hence, considering the existence of high probability mutation hotspots in the dystrophin genome and the functionality of truncated dystrophin derivatives, the application of ODNs to induce specific exon skipping, blocking the splicing machinery of the affected exon while restoring an in-frame function, is a promising approach (Pramono *et al.* 1996).

The possibility of using circulating cells, capable of homing naturally to the site of lesions, to deliver therapeutic substances is at an early stage of investigation. In terms of DMD, macrophages naturally locate to sites of severe muscle necrosis in dystrophic muscle, hence genetically engineering muscle-specific macrophage populations to direct dystrophin expression at affected sites enables the possibility of targeting triggered by the pathology itself. Preliminary studies have reported the successful homing of engineered macrophages to sites of muscle damage for periods as long as two months (Parrish *et al.* 1996).

An alternative approach to the genetic complementation of dystrophin in DMD is the possibility of the upregulation of the expression of the functionally similar DRP, utrophin, in dystrophic muscle. Utrophin, an autosomal homologue of dystrophin, is a ubiquitously expressed 395 kDa protein with 88% amino-acid sequence homology to dystrophin, containing all four structural domains (Blake *et al.* 1995). Utrophin is believed to be a foetal form of dystrophin, with a large number of functional and structural similarities, which not surprisingly allow its association with a related DAGC in non-dystrophin expressing cells. The expression of utrophin is believed to precede that of dystrophin, but is later developmentally replaced with dystrophin in muscle until its only site of expression is at the NMJ, where it interacts with the actin-based cytoskeleton (Tinsley *et al.* 1994). Hence, considering the

high level of homology between the two proteins and the abilities of both to bind the DAGC, the possibility of the functional replacement of dystrophin with utrophin in all relevant muscles is a probable option. A recent study of the gene transfer into mdx mouse of a truncated utrophin transgene resulted in high-level expression in skeletal and diaphragm muscles, accompanied by marked reductions in the dystrophic pathology and restoration of the DAGC, with no deleterious effects (Tinsley *et al.* 1996). However, the possibility that utrophin is less adapted to deal with mechanical stress than dystrophin, together with the finding that utrophin and dystrophin binding of F-actin are regulated to different extents by calcium/calmodulin, suggests that overexpression of utrophin as a potential therapy for DMD and BMD may be oversimplified (Winder and Kendrick-Jones 1995)

13.10 CONCLUSIONS

To date experimental gene transfer studies in mdx mouse have exemplified the possibilities of dystrophin gene transfer to skeletal muscle, exhibiting efficient, stable and long-term expression and reversal of the dystrophic pathology of the murine model in many studies. However, many unresolved issues remain, which rightly preclude the human applications of this technology. Direct questions concerning the dosage, route of administration, safety limits and physiological effects of dystrophin gene transfer require elucidation, which may be approached through scaled up studies in larger models such as the xmd dog. In addition, more fundamental studies into the precise congenital functions of dystrophin and the DAGC, as well as our ever-increasing understanding of the biology of muscle, will greatly shape the structure of future therapies. It is inevitable that many of the present obstacles facing gene therapy for DMD are likely to be resolved with the technical advances in gene transfer technology such as vector stability, efficiency, levels of expression and immunological barriers, enabling the translation of our understandings into effective clinical therapies and prognostic tools. Besides the applications to DMD, the unravelling of the dystrophin story will greatly aid the cellular and biological knowledge towards therapies for the many related autosomal muscular dystrophies, as well as many other currently untreatable disorders.

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14 Lysosomal Storage Disorders

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14.1 INTRODUCTION

Lysosomal storage disorders (LSD) are a complex group of diseases both at a clinical and a cellular level. There are approximately 50 diseases that are characterised by an inability of lysosomal hydrolytic enzymes to degrade macromolecules into smaller products or by the failure to transport these products into the cytosol. Progressive accumulation of either undegraded or partially degraded substrate produces massive lysosomal distension, anatomical distortion and eventual functional impairment in affected cells. The clinical problems that arise include severe bone abnormalities such as kyphoscoliosis and atlantoaxial instability in the mucopolysaccharidoses, neurodevelopmental regression and inanition in some mucopolysaccharidoses and in the leukodystrophies, and bone marrow failure in the most common LSD, Gaucher's disease. (See Table 14.1 for a summary of the clinical features of the LSDs discussed in this chapter.)

A feature of many LSDs is that even within a single enzyme disorder, there may be substantial variation in the age at which the disease becomes clinically detectable, in the rate at which functional deterioration occurs and in the range of affected tissues. As a general principle, disease presenting in infancy or early childhood is associated with a more rapid progression of symptoms and a poorer prognosis, while later onset disease is often compatible with a relatively normal lifespan. These clinical features define many of the issues facing a systematic approach to genetic therapy in LSD, and can be summarised as the need to correctly identify patients with a particular prognosis at an age at which any pathological changes are still amenable to therapy, and to successfully restore enzyme levels in a broad range of tissues, including the central nervous system (CNS), for specific LSDs.

Table 14.1 Summary of clinical features of the major LSDs discussed to in this chapter

Mucopolysaccharidosis

A group of 11 disorders accounted for by failure of lysosomal degradation of the glycosaminoglycans heparan, dermatan and chondroitin sulfate
MPS-IH (Hurler's syndrome)

Largely autosomal recessive although MPS-II is X-linked

MPS-IH/S (Hurler's syndrome/
Scheie's syndrome) MPS IS (Scheie)
MPS-IS (Scheie's syndrome)
MPS-II (Hunter's syndrome)
MPS-III A–D (Sanfilippo's syndrome)
MPS-IV A&B (Morquio's syndrome)
MPS-VI (Maroteaux–Lamy syndrome)
MPS-VII (Sly's syndrome)

Wide variation in phenotype but principal tissues affected are bone, particularly axial skeleton, brain (MPS-IH, MPS-II, MPS-III, MPS-VII and MPS-VIII), soft tissues, liver and spleen
Bone marrow transplant helpful in certain subgroups provided undertaken before neurological deterioration

MPS-VIII (Keratan and Heparan sulfaturia)

Disorders of glycoprotein degradation

Six disorders resulting from the failure of lysosomal degradation of oligosaccharide

α -Mannosidosis

β -Mannosidosis

Fucosidosis

Sialidosis

Aspartylglucosamine

Carbohydrate deficient glycoprotein syndrome

All disorders are autosomal recessive

Considerable variation in severity of disorder within an enzyme deficiency but clinical features include progressive mental retardation and bone abnormalities (α -mannosidosis, fucosidosis, sialidosis), epilepsy and spastic quadriplegia (β -mannosidosis)
There is no definitive therapy

Gaucher's disease

Eponym refining to a clinically heterogenous group of disorders, resulting from lysosomal accumulation of glycosylceramide. Predominantly due to mutations in the lysosomal hydrolase acid β -glucosidase but mutations in the activator protein posaposin are also described.

Autosomal recessive

Wide variation in phenotype largely differentiated by neuronopathic and (non-neuronopathic forms. Other tissues affected are liver, spleen, bone marrow and bone

Enzyme replacement useful in non-neuronopathic disease, where bone marrow transplant is also curative

Table 14.1 (cont.)

<i>Metachromatic leukodystrophy</i> Disorder of myelin metabolism characterised by accumulation of cerebroside sulfate and due to abnormalities of the enzyme arylsulfatase A (principally) or the protein activator, saposin B	Both disorders are autosomal recessive Wide variation in rate and severity of phenotype, which is characterised by neurodegeneration Bone marrow transplant has been tried but is of questionable benefit unless patients are asymptomatic
<i>Krabbe's disease</i> Disorder of myelin metabolism resulting from a deficiency in the enzyme cerebrosidase (galactosylceramide β -galactosidase)	Autosomal recessive Rapidly fatal with increasing spasticity, blindness and deafness There is no therapy

14.2 DEFINING THE PATIENT POPULATION

14.2.1 THE RELATIONSHIP BETWEEN GENOTYPE AND PHENOTYPE

It is usual to investigate an experimental treatment such as gene therapy in patients where one can predict a poor outcome and for whom there are no other useful therapies. Given the wide variation in phenotype within a single LSD, this can present a major challenge. To some extent this issue is being slowly resolved as a better understanding of the relationship between genotype and functional abnormality is established. For example, the LSD metachromatic leukodystrophy (MLD) most commonly arises because of a primary deficiency of the enzyme arylsulfatase A (ARA), although a deficiency in the ARA activating protein, saposin B, has also been identified. As ARA is important for hydrolysing sulfatide from cerebroside-3-sulfate (a major constituent of myelin sheets), a reduction in enzyme activity results in accumulation of the sphingolipid and produces functional impairment in oligodendrocytes and Schwann cells. As for many LSDs, variation in the rate of progression is clearly apparent and three patterns of MLD are recognised: early onset (within the first year of life), juvenile (usually symptomatic in the latter part of the first decade) and an adult form which begins in the second decade. They all share similar clinical features, including progressive quadriparesis, pyramidal and extrapyramidal signs, dementia and a detectable decrease in nerve conduction velocities.

For a substantial number of patients with MLD the age of onset and the rate of clinical progression are strongly correlated with the underlying genotype, which in turn predicts for the level of functional enzyme [1,2]. Al-

though almost forty mutations have been identified in the ARA gene, two of these account for 50% of patients. These are the loss of a splice donor site at exon 2 and an amino acid substitution (P426L) producing an unstable but functional enzyme. Homozygotes for splice mutations and deletions that do not produce functional enzyme, progress early and severely as the infantile form of the disease. As little as 1–5% of the normal enzyme level can significantly ameliorate disease progression, and in MLD 1–5% of normal enzyme levels may give rise to 30% of normal degradative activity, resulting in the juvenile form of the disease. Such an intermediate level of enzyme activity commonly arises from the combination of a null mutation and a second mutation producing an unstable but active ARA. Homozygotes for mutations causing unstable enzyme usually have measurable sulfatide degradation of around 50% of normal and manifest late-onset MLD. This relationship is not absolutely predictive, with some unexpected and so far unexplained genotype–phenotype relationships.

Similar relationships between genotype and phenotype are being uncovered for other LSDs, including the most common mucopolysaccharidosis, α -L-iduronidase deficiency (MPS-I; type I mucopolysaccharidosis; Hurler's Syndrome), as well as the sphingolipidosis, Gaucher's disease [3,4]. In MPS-I homozygotes for the non-sense mutations W402X and Q70X have no residual enzyme activity, and progress early with bony, soft tissue and CNS disease (MPS-IH). Patients with low but detectable enzyme levels (1–5%) have an enormous variation in phenotype, with some patients having no CNS problems, less-obvious soft tissue abnormalities and a normal lifespan (MPS-IS or MPS-IH/S). Many of these patients are compound heterozygotes for the null W402X mutation. Unravelling this area is a complex task as over 50 different mutations in the iduronidase gene have been identified and the genetic diversity observed presumably accounts for the wide variation in clinical phenotype. Although some headway is being made from understanding the nature of the mutations, it is still difficult to reliably predict for disease severity. This is a recurring theme within the LSDs, but it should be hoped that a better understanding of the interaction of alleles in compound heterozygotes and of the interaction of polymorphic genes with mutated alleles will eventually lead to an earlier and more reliable prediction of the clinical course in the majority of disorders.

At a pragmatic level there are already a modest number of diseases where a specific genotype can reliably predict for clinical outcome, making it possible to identify candidates for genetic therapy. These include homozygosity for W407X and Q70X in MP-IH, N307S for type I Gaucher's disease and L44P in individuals with appropriate ancestry in type III Gaucher's disease.

14.3 STANDARD AND 'EXPERIMENTAL' TREATMENTS

14.3.1 SUPPORTIVE CARE

The second issue that defines this patient population is the absence of any other useful therapy. At present this is a relatively simple issue in LSD. There are three forms of treatment: (i) supportive care, i.e. managing symptoms and maintaining quality of life, and enzyme replacement either (ii) through repeated exogenous administration of an enzyme or (iii) through transplantation of tissues that are capable of secreting and transferring enzyme. Supportive care has a role in all patients whether or not any other treatments are added. For example, Gaucher's disease can be painful when bony crises supervene, and individuals may require good analgesic support; patients may require splenectomy for massive organ infiltration, and transfusions can be helpful in reducing the lassitude and breathlessness of profound anaemia. In other disorders, such as the mucopolysaccharidoses, careful attention to spinal instability or structural kyphoses, surgical intervention in carpal tunnel syndrome, and investigation and support of hearing or ocular abnormalities will all prolong and enhance the quality of a patient's life.

14.3.2 ENZYME REPLACEMENT

For a number of lysosomal storage disorders there is the additional prospect of therapy through enzyme transfer and replacement in affected tissues, either through administration of an exogenous enzyme or through transplantation of a tissue source. These strategies stem from the observations that many lysosomal enzymes can be secreted and then sequestered by lysosomes in distant tissues and can also be transferred to neighbouring cells by direct cellular contact. Lysosomal proteins are first synthesised in the rough endoplasmic reticulum, transported into the Golgi cisternae following cleavage of a signal peptide and transferred to the acidic prelysosomal and lysosomal compartments (reviewed in [5]). At the point of transfer from the Golgi apparatus, proteins are segregated either for immediate transport to the lysosomal compartment or into a secretory pathway which leads to release of protein from the cell and availability for re-uptake via receptor-mediated endocytosis in other tissues (reviewed in [6]). The principal mechanism for uptake is through the widely expressed mannose-6-phosphate receptor (M6P-R) although other receptors are also implicated. These include the mannose and *N*-acetylglucosamine receptors of macrophages and monocytes, possibly sialic acid receptors expressed on glial cells, asialogalactose-containing proteins on hepatocytes and receptors for fucose residues on fibroblasts (reviewed in [7]). Studies in a number of animal species indicate that the M6P-R is developmentally regulated, with highest levels expressed

during embryogenesis [8–10]. During the postnatal period M6P-R expression is rapidly downregulated, but levels remain sufficiently high to facilitate endocytosis of lysosomal enzymes in a variety of tissues, including neurons and glia. Under normal conditions the secretion and subsequent recapture of enzymes does not contribute significantly to the total lysosomal enzyme level in cells, although it has been shown that this pathway is able to contribute up to 12% of the total lysosomal enzyme complement of cultured human fibroblasts [11,12].

This process has been successfully exploited for treatment of Gaucher's disease, which arises from a deficiency in the enzyme glucocerebrosidase, which hydrolyses the membrane protein glucosylceramide to glucose and ceramide. The major source of substrate is the cellular membranes of haemopoietic cells and this defines the major pathology of the disorder, which is lysosomal distension in tissue macrophages. Gaucher's disease has all the typical clinical features of an LSD, including early and late forms and a wide phenotypic expression. However, the most prevalent form of the disease is the milder form (type I, chronic non-neuronopathic), which principally involves the reticuloendothelial system and spares the CNS.

Native enzyme was originally prepared for clinical use in the late 1960s and early 1970s. Although it was possible to show some clinical effect, results were inconsistent and this was attributed to hepatic sequestration of enzyme [13,14]. As liver pathology is not a major feature of Gaucher's disease the enzyme was modified in order to specifically target tissue macrophages. This was accomplished by the sequential deglycosylation of purified native enzyme extracted from human placenta, producing a modified enzyme with exposed terminal mannose residues [15] and which targeted the mannose lectin on macrophage plasma membranes. Excellent clinical responses have been obtained using the modified glucocerebrosidase with regression of splenomegaly and improvement in haematological parameters so that enzyme replacement is proving highly beneficial in an important subset of patients affected by type I Gaucher's disease [16,17].

Based on these successes, recombinant enzyme has been prepared for preclinical assessment in a number of LSDs including MPS-I, MPS-II, MPS-VI and MPS-VII [18–21]. Putting aside logistical problems such as the supply of enzyme and the high cost of treatment, the major clinical issues in extending this approach to other LSDs come from the following considerations. Firstly, the symptoms of the prevalent form of Gaucher's disease can be controlled by targeting enzyme to a single system. Reversing other multiple tissue pathologies may be more difficult particularly in the CNS, where there has been little evidence of therapeutic effect in the neuronopathic forms of the disease. Secondly, there is evidence of residual enzyme activity in all patients with Gaucher's disease, and by implication, patients with Gaucher's disease have acquired tolerance to the functional protein during embryogenesis. In a

number of other severe forms of LSD which are not lethal during foetal development affected individuals have no residual enzyme activity at birth and may not prove immunotolerant of exogenous enzyme. This includes α -L-iduronidase deficiency (MPS-I), where injection of the recombinant enzyme in a null adult dog model demonstrated that the exogenous enzyme did not reverse pathological changes in the CNS, cornea, heart valves or articular cartilage [22]. One possible explanation was that all affected dogs showed evidence of an immunological response to administered enzyme with deposition of immune complexes in kidneys during the first year of treatment. Problems with an immune reaction to administered enzyme have also been reported in a feline model of MPS-VI (Maroteaux-Lamy Syndrome) [23]. If similar problems arise in other animal LSDs and in the equivalent human disorders then it is likely that enzyme replacement may prove ineffective for patients with undetectable enzyme activity and, by extension, the most severe forms of disease.

14.3.3 BONE MARROW TRANSPLANT

The second approach to providing treatment is through transplantation of tissues capable of secreting enzyme. This has largely been undertaken through bone marrow transplant (BMT) with matched related allogeneic grafts and, more recently, by cells derived from matched unrelated donors. One advantage of BMT over exogenous enzyme replacement is that transplantation should provide permanent reconstitution of enzyme activity both for disorders which primarily affect the haemopoietic system such as type I Gaucher's disease and to cells outside the haematopoietic compartment by continuous secretion and recapture of enzyme. A unique advantage of BMT is that BMT-derived lymphocytes and macrophages are efficient donors of enzyme through direct cellular contact with neighbouring cells. The cells of the haematopoietic system are able to infiltrate and provide enzyme in sites such as the CNS which may not be accessible to transfused enzyme.

Initial studies in animal models of LSD such as mannosidosis, fucosidosis, MPS-VII (Sly's syndrome) and Krabbe's disease have shown very encouraging results, including the reversal or halting of neurological disease [24-27]. The results from human studies are more variable. A recent review of the European allogeneic BMT experience in LSD confirmed that BMT was a useful treatment in non-neuronopathic Gaucher's disease, where all of five patients treated with type I disease showed clinical regression in signs and symptoms [28]. Patients with established CNS pathology, including six patients with MLD, did not obtain any benefit from transplantation. Stabilisation of disease was seen in the mucopolysaccharidoses MPS-I and MPS-II. The reason for these disappointing results may stem from the variation in clinical severity of affected patients, as well as differences in disease types.

Other single-institutional studies have shown that there can be a clear clinical benefit from transplant in specific disorders such as MSP-I, but patients must be transplanted early before there is significant functional impairment in the CNS. Under these conditions neurological deterioration can be either prevented or slowed [29].

To date bone marrow transplantation can only be recommended as a partially effective treatment for the LSDs (see Table 14.1). While transplantation offers some hope of slowing disease progression, it is limited by the risks of profound immunosuppression in non-life-threatening but debilitating conditions such as the milder phenotype of Gaucher's disease, and by lack of suitable donors in more severe conditions. In an attempt to increase the availability of transplants for patients without matched related sibling donors, a number of transplantation units have explored the use of matched unrelated donor transplants in patients with severe disease. Of these the North American experience of matched unrelated donor transplantation in MPS-IH (severe phenotype) has been reported and confirms the high morbidity and mortality of the procedure, with 50% of patients dying in the first two years following transplantation, and a high incidence of chronic graft versus host disease [30]. This issue is in part a circuitous problem, as the more severe the phenotype the earlier the need for a transplant, and the less clinical latitude to use optimal conditioning regimens because of their attendant toxicity. Moreover, the process of chronic graft versus host disease involves the CNS and can compound the neurological damage produced by lysosomal storage.

In summary, there is a clear potential for defining candidate patients for genetic therapy by mutational analysis prior to onset of their disease. This is most firmly established for patients who are homozygous for mutations which do not give rise to any functional protein. BMT has been assessed for a variety of LSDs and is particularly effective in some diseases, including type I Gaucher's and MPS-IH, but is limited by the availability of suitable donors and the collateral toxicity of the procedure. Consequently, this approach is usually only offered to patients with poor risk disease. For other patient groups in whom there is no clinical evidence of an effect from BMT or from enzyme replacement, new treatment strategies are required.

14.4 TISSUE TARGETS FOR GENE THERAPY

The first landmark for undertaking genetic manipulation of autologous tissues in LSD has been reached by the cloning of most of the genes. Moreover, the cDNAs for many of the LSDs are less than 5 kb in size and thus available for insertion in the first generation of retroviral-based gene therapy vectors. Animal models have been developed for a substantial proportion of

the disorders although not all are widely available (Table 14.2). These provide a useful system in which to assess therapeutic strategies.

14.4.1 TRANSPLANTS OF 'NEO-ORGANS'

The initial attempts at genetic therapy are built on the phenomenon of lysosomal cross-correction described earlier in this chapter and on the clinical results of allogeneic bone marrow transplantation. Fortunately, these approaches are amenable to testing with the first generation of vectors available for clinical use. Moullier and colleagues have concentrated on utilising the secretory pathways for lysosomal cross-correction through creation of a surgically implanted neo-organ containing transduced skin fibroblasts, and have applied the technique to production of both β -glucuronidase (MPS-VII) and α -L-iduronidase (MPS-I) [31,32]. The investigators assembled a matrix containing polytetrafluoroethylene fibres coated with basic fibroblast growth factor, and implanted it into the peritoneal cavities of β -glucuronidase-deficient mice. The matrix became vascularised and was capable of acting as a receptacle for transduced skin fibroblasts expressing the β -glucuronidase gene. In these experiments β -glucuronidase expression was demonstrated from the implanted neo-organ over a period of 3 months. Enzyme was invariably found in the liver of transplanted mice, and this phenomenon reversed on surgical removal of the neo-organ. This indicated transfer of activity by a secretory process rather than migration of cellular constituents of the neo-organ into the liver. Activity was also detected in the lungs of animals, although at a lower frequency (two out of three treated animals), but not reliably in spleen and not in nucleated blood cells. More exhaustive studies in the MPS-VII mouse by the same group subsequently confirmed the ability to cross-correct lysosomal storage in the liver and spleen of young adult (4–8 weeks) mice, and demonstrated that the enzyme was predominantly localised to tissue macrophages [33]. Unfortunately, the levels of enzyme reached in peripheral tissues were 0.5–6% of those of phenotypically normal carrier littermates and with substantial variation between treated animals. Although the enzyme does not cross the blood–brain barrier, occasional scattered cells that were focally positive for β -glucuronidase were found in the CNS and this is presumed to represent ingress of macrophages bearing enzyme. The trace levels of enzyme detected in brain tissue were insufficient to produce any major change in the neurological abnormalities in the mice. A second feature of the study was that over the time period of the initial experiment the majority of animals developed antibodies to the human β -glucuronidase enzyme, although this effect was largely preventable by concurrent immunosuppression.

It is not possible to directly compare the outcome of these experiments

Table 14.2 Animal models in lysosomal storage disorders

Group	Specific disorder	Enzyme deficiency	Animal model	Reference
Mucopolysaccharidosis	MPS-I	α -L-iduronidase	Cat	83
			Dog	84
	MPS-III	<i>N</i> -acetylglucosamine-6-sulfatase	Goat	85
	MPS-VI	Arylsulfatase B	Cat	86
	MPS-VII	β -glucuronidase	Dog	87
			Mouse	88
Glycoprotein degradation and structure	α -Mannosidosis	α -Mannosidase	Cattle	89
			Cat	90
	β -Mannosidosis	β -Mannosidase	Goat	91
			Cattle	92
			Dog	93
Cellular cholesterol lipidosis	Fucosidosis	α -L-fucosidase	Dog	94
	Niemann-Pick disease type C	Unknown	Mouse	94
Sphingolipidosis	Gaucher's disease	Acid β -glucosidase	Knockout mouse	95
Galactosylceramide lipidosis	Krabbe's disease	Galactosylceramidase	Mouse	96
			Dog	97
			Sheep	98
			Monkey	99
G_{M1} gangliosidosis	β -Galactosidase deficiency	Acid β -galactosidase	Cat	100
			Dog	101
			Sheep	102
			Calf	103
G_{M2} gangliosidosis	Sandhoff's disease	β -Hexosaminidase B	Cat	104
	G_{M2} activator deficiency	G_{M2} activator protein	Dog	105

with the nearest equivalent therapy, i.e. systemic administration of recombinant enzyme, as this study with enzyme replacement was undertaken in immature animals, which are likely to have higher levels of M6P-R expression. However, it is notable that when young MPS-VII mice (age 0–6 weeks) were administered recombinant enzyme in a single or multiple dosage schedule, much higher levels of transferred enzyme were recorded following multiple dosing in a variety of organs, including up to 35% of normal enzyme activity in the liver and 6% of normal activity in the brain [34]. The beneficial effects in terms of reduced lysosomal storage were seen in a broad range of tissues, including neurons, although not in the glial population. Given that a multi-dosing schedule was more effective in young MPS-

VII mice, it may be that sustained expression of enzyme from an implanted tissue source such as the neo-organ described by Moullier and colleagues will be beneficial if surgery is performed early in the disease process. This is under investigation and the results of these experiments are awaited.

14.4.2 MANIPULATION OF PRIMARY LYMPHOCYTES

A second tissue source which may be useful for protein production and transfer is the primary human lymphocyte. Although B and T lymphocytes secrete only small amounts of lysosomal protein they are capable of transferring functional enzymes by direct cellular contact [35]. These observations were first made in 1981, when lymphocytes were shown to cross-correct fibroblasts in patients with the LSD MPS-VII (Sly's syndrome) [36]. It was demonstrated that transfer of the β -glucuronidase could only take place when cells were in direct contact both by physically separating cellular populations and by manoeuvres which blocked cell contact. This facility was not universally applicable to all LSDs, with a notable exception to enzyme transfer being Gaucher's disease [37]. Two forms of the α -D-mannosidase enzyme that are associated with the endoplasmic reticulum and Golgi compartments were also not transferred. However, a high molecular weight precursor form of the enzyme, which can be correctly processed, is transferred and is ultimately capable of functional cross-correction [38]. In addition to β -glucuronidase the enzyme iduronate-2-sulfatase has also been shown to transfer to and correct fibroblasts from patients affected by the appropriate LSD [39].

Braun and colleagues are building on these observations and exploring the ability of autologous, genetically manipulated lymphocytes to correct the functional abnormalities in patients with a mild form of the LSD iduronate-2-sulfatase deficiency (MPS-II, Hunter's syndrome). Using an amphotropic retroviral vector L2SN, they have demonstrated high-level expression of iduronate-2-sulfatase in lymphoblastoid cell lines derived from patients with MPS-II [40]. These cells have been co-cultured with fibroblasts from patients and have corrected the metabolic abnormality.

Protocols for lymphocyte isolation and expansion are well established, and genetically modified lymphocytes have been successfully used in a clinical study for treatment of the primary immunodeficiency, adenosine deaminase deficiency [41]. With this background Whitley and colleagues have gone on to produce a clinical protocol which will assess the value of genetically modified autologous lymphocytes in MPS-II [42]. However, as lymphocytes are not a major constituent of CNS tissues, it is not expected that this approach will be beneficial to LSDs that have a major neurodegenerative component, and thus the group is confining the approach to patients who have a mild phenotype with no CNS involvement.

14.4.3 AUTOLOGOUS BONE MARROW

As bone marrow transplantation has been found to be a useful approach in certain LSDs it is perhaps not surprising that there has been considerable interest in pursuing this as an alternative route for enzyme production through genetic manipulation of autologous bone marrow. The approach has the advantage of extending the availability of bone marrow transplantation to patients without an allogeneic sibling donor, or where a matched sibling donor is a heterozygote for the disorder and produces low enzyme levels. The strategies of using matched unrelated donors for transplantation is only available for certain racial groups because of the unbalanced racial mix in donor registries, and, as has been discussed, carries a higher morbidity and mortality, particularly in very young children. One interesting experimental observation is that engraftment of autologous haemopoietic stem cells can take place without initial conditioning, although a degree of conditioning certainly facilitates the level of engraftment [43,44]. The potential for less intensive conditioning, absence of graft versus host disease, and the consequent reduction in transplant associated morbidity and mortality might extend the opportunity of corrective bone marrow transplant to very young children in the first few months of life and also to patients with a milder but still chronically debilitating disease.

Sources of autologous cells for long-term haemopoietic reconstitution can be found at low levels in bone marrow, and in higher numbers in blood following cytokine mobilisation, or at birth in umbilical cord blood. Methods for establishing efficient transduction of these cells using retroviral vectors have largely been established, although issues regarding the impact of the transduction protocol on accelerating lineage commitment during gene transfer have not been adequately addressed. Many investigators are choosing to test the value of cytokine mobilised progenitor cells as primary targets for gene transfer, as they are present in higher numbers in blood compared to bone marrow, and also appear to be cycling more rapidly, facilitating retroviral transduction. The 'gold standards' for assessing successful gene transfer to a subset of primitive progenitors capable of long-term bone marrow reconstitution are, firstly, the ability of transduced cells to successfully rescue lethally conditioned recipients following myeloablative chemotherapy or irradiation, or secondly the demonstration of persistent gene transfer and expression in long-term *in vitro* models of haemopoiesis.

The first of these is applicable as part of the preclinical evaluation of gene therapy when there are suitable animal models for assessment of the clinical protocol (see Table 14.2). However, because of the necessity to demonstrate long-term persistence of gene expression in human cells, the second method is also used. The best *in vitro* model for testing transduction of long-term bone marrow repopulating cells comes from a modification of

the Dexter long-term culture system, where human bone marrow can be maintained in culture for up to 6 months. In the first 5–6 weeks of culture committed progenitors undergo clonal extinction. Further haemopoietic activity results from very early progenitors and can be investigated through a variety of colony forming assays. This model has been adapted for measuring gene transfer and expression in progenitors mobilised into peripheral blood, as well as those obtained from umbilical cord blood, by providing an established stromal support layer. The long-term culture system has also been used to investigate the factors influencing retroviral gene transfer and expression in progenitor cells obtained both from peripheral blood and from bone marrow in patients with Gaucher's disease [45]. Both bone marrow and peripheral blood progenitors were examined for transfer and stable transduction of colony forming cells following exposure to a retroviral vector carrying the glucocerebrosidase gene. A combination of the cytokines, interleukin-3 (IL-3), IL-6 and stem cell factor, together with stromal support, gave the best transduction levels at 5 weeks in continuous culture. The effects of stroma and cytokine combination on efficiency of gene transfer were most pronounced for peripheral blood progenitors, where up to 70% of colonies were positive for glucocerebrosidase at 5 weeks.

Similar experiments have been undertaken by Fairbairn and colleagues for the α -L-iduronidase gene (MPS-I) [46]. This group demonstrated excellent production of α -L-iduronidase in long-term cultures for up to 6 months, at which point cultures had become senescent. Moreover, they were able to demonstrate high levels of gene transfer to primitive progenitors without using either allogeneic stroma, growth factors or intensive transduction schedules. While it is not clear why this group has been particularly successful in obtaining high levels of gene transfer without the addition of cytokines or intensive manipulation of cells, this may be a major advantage and an important factor in preventing premature lineage commitment before successful gene transfer has occurred. At a functional level α -L-iduronidase was shown to be secreted into the medium, and correction of lysosomal storage was demonstrated both histologically and through reduction in S³⁵ labelling of cells.

These studies are good preclinical evidence for moving forward into clinical trials in the respective disorders, and Lashford and colleagues have a clinical trial open for investigating genetically manipulated bone marrow as treatment for MPS-IH. In the case of Gaucher's disease further supportive data is provided from animal studies [47,48]. These show that murine bone marrow can be transduced at high efficiency by retroviral vectors (MFG and LN series) containing the human glucocerebrosidase gene and that these cells are capable of reconstituting lethally irradiated recipients. High levels of glucocerebrosidase activity were found in tissue macro-

phages following bone marrow transplantation and gene expression has been sustained in mice for up to 7 months [48]. The kinetics of glucocerebrosidase expression in the macrophage population has also been studied in detail. Using a slightly different retroviral vector (N₂) Krall and colleagues transplanted transduced bone marrow into lethally irradiated mice [49]. At early time points (1–2 months after transplant) donor cells were found predominantly in the spleen, blood, bone marrow and thymus of recipient mice. With the passage of time donor cells were in evidence in other tissues, including the CNS. For a tissue such as liver the population of donor macrophages increased from 10% of cells at 1 month to 70% at 6 months. A similar rise was seen in the microglial population of the CNS, reaching approximately 20% of cells by 6 months, with a largely perivascular distribution. Given the successful repopulation of the CNS by a donor microglial population, the critical question for genetic therapy of LSD with a neurodegenerative component is why the clinical effect of bone marrow transplantation is so variable.

There are likely to be a number of contributing factors, including the degree of pre-existing damage (age- and disease-related), enzyme levels in the donor (heterozygote or homozygous sibling), nature of the conditioning regimen (age-related and physician choice) and level of M6P-R expression in tissue constituents (age-related) [50]. Animal studies *in vitro* and *in vivo* demonstrate the capacity for lysosomal proteins to be transferred to both neurons and glia [51,52]. A seminal study by Walkley and colleagues in a feline model of α -D-mannosidosis indicated that transplantation of bone marrow from phenotypically normal littermates into affected kittens could result in 9–40% of normal enzyme activity within the CNS [24]. Highest levels of mannosidase were once again associated with the perivascular microglia, but enzyme was also demonstrated in neurons and other glial populations. It may be important that studies investigating the value of BMT in correcting tissue abnormalities have largely utilised myeloablative radiation as a conditioning regimen [53,54]. In two single studies where the relationship between radiation and subsequent enzyme level, following BMT in mice, was investigated there was a clear relationship between increased radiation dose and a better level of enzyme expression in all tissues examined [55,56]. This may reflect either better engraftment or a response to tissue injury as higher radiation doses were also associated with increased toxicity both in the CNS and in the skeleton of young animals. Consequently, one major concern is that the perceived clinical benefit of a reduction in conditioning for autologous reconstitution by genetically modified bone marrow may be outweighed by poorer tissue levels of enzyme production, as well as an enhanced immune response to protein in patients with a null phenotype.

14.4.4 DIRECT TARGETING OF THE CNS

There are two major strategies for improving gene transfer and expression in the CNS: (i) transduction of cells with neurotropic vectors and (ii) providing intracerebral cellular grafts. The retroviral vectors that have proven useful for the first gene therapy experiments have little role to play in the CNS, since they are not capable of transducing in the post-mitotic cells that make up the mature CNS.

Two viruses which have come to prominence as potential vectors for gene delivery to the CNS are the adenovirus and herpes simplex virus (HSV). Of these HSV is perhaps the most easily recognisable as a potential vector in the CNS. HSV has the capacity to infect and replicate in most cell types but with collateral cellular lysis [57]. However, when peripheral neurons are infected, the virus does not lyse cells but establishes a latent state, in which the viral genome persists as a nuclear episome [58]. The principal sites of latent infection are the dorsal root ganglia, and this tissue specificity is attributed to expression of a cell-specific transcription factor, OCT-2, which binds the early gene promoter of the viral DNA [59]. HSV-based vectors aim to capitalise on the neuronal tropism and latency of the wild-type virus. However, the main problem in creating such a vector revolves around the concerns regarding the clinical risks if latency is lost and subsequent reactivation of the lytic life cycle within the CNS occurs. A second problem comes from identifying a suitable promoter which is capable of achieving appropriate levels of therapeutic gene expression.

In the first scenario the reactivation of an HSV vector in brain may produce a life-threatening encephalitis. When high titres of a wild-type HSV have been injected into the cerebral cortices of animals, infected subjects have rapidly succumbed to a viral-induced encephalitis [60]. To produce a disabled vector a number of recombinant viruses have been developed, and these include those in which the viral gene thymidine kinase (TK), which produces an enzyme that is essential for viral growth in neurons, has been removed. An alternative strategy is the removal of the immediate early (IE) gene sequences. Experiments investigating the first strategy deleted the viral TK, and the cDNA of the purine salvage enzyme, hypoxanthine phosphoribotransferase, was inserted. Administration of the vector into the brains of mice still resulted in significant toxic effects in the CNS and there is nothing to suggest that this is a safe viral vector for human studies [61]. The second approach requires the provision of the IE gene functions from an exogenous source [62]. This has been achieved by growing the disabled virus in cultured cell lines, which have been artificially engineered to express the IE genes. Unfortunately, this results in a risk of regenerating an intact virus through recombination events between the defective virus and the genetic sequences in the cultured cells, and while this

is likely to occur at low frequency, it once again produces a vector that is not acceptable for human use. A further approach is to develop episomal HSV-based vectors that contain all the *cis*-acting sequences required for replication and packaging of their DNA into virions, but that can only be stably propagated in the presence of wild-type virus, which provides the transactivating viral proteins required for these processes [63,64]. Unfortunately, all of the final viral stock is a mixture of packaged vector and wild-type HSV, with potential for producing the toxic effects of pure wild-type virus. While there are obviously strategies for producing defective helper viruses these have not been developed to a level at which they are safe for human experimentation [65].

Apart from the major issue of safety, the second problem associated with the generation of HSV vectors has been the identification of suitable promoters for long-term gene expression in a vector that is transcriptionally inactive in its latent state. Those vectors that have utilised the early activating genes require the production of an IE gene product for high-level activity, and run the risk of activation of lytic infection and subsequent toxicity, whereas the IE promoters are specifically repressed in latently infected neuronal cells, and are thus unable to sustain long-term expression of genes of interest. A clear choice is the LAT promoter, which is transcriptionally active in latently established infection. Using a LAT promoter, long-term expression of a reporter gene has been demonstrated in the peripheral nervous system, and HSV-LAT mediated expression of the lysosomal enzyme β -glucuronidase has been achieved in the CNS of MPS-VII mice for up to 4 months, albeit in relatively localised areas [66].

In view of these problems, a number of groups have turned to an alternative vector system, the adenovirus, which despite its usual epithelial tropism has the ability to infect the CNS [67]. Like HSV adenovirus is capable of infecting post-mitotic cells and establishing itself as an episome. Vectors have been developed that have removed the IE genes, rendering the virus incapable of self-replication. Removal of additional constituents within the viral genome, such as the E3 or E5 region, has created sufficient capacity to insert foreign genes. With respect to the LSDs, adenoviral vectors have been created to carry the MLD gene, ARA, and this enzyme has been successfully expressed *in vitro*, both in fibroblasts and in the ultimate target tissue, oligodendrocytes [68,69]. These are early studies and there is no information on the longer term expression and effect in appropriate animal models. To date, adenoviral-mediated gene expression of reporter genes has indicated that protein expression in the CNS is relatively transient [70].

Unfortunately, there are some concerns regarding the toxicity of adenovirus. A number of studies in mice and primates have demonstrated a significant host immune response to the vector [71–73]. This effect is attributed to loss of E3 function, which in the intact viruses modulates cellular

expression of major histocompatibility complex (MHC) class I antigens and thus dampens the host immune response to infecting virus [74]. Consequently, there is a considerable amount of ongoing work investigating removal of other elements of the viral genome to produce a safer and less immunogenic system [75,76].

To date, the majority of studies of adenoviral vectors bearing marker genes have been undertaken either *in vitro* or by direct inoculation of the vector into the cerebrum. It would seem that following direct injection there is a relatively limited transfer of genetic material around the injection site, without the whole CNS becoming infected and transduced by the viral vector [77]. This is likely to cause practical problems for diseases where widespread delivery of DNA is necessary. Investigators have looked at disrupting the blood-brain barrier by osmotic agents, injecting the adenovirus into the bloodstream and following its fate within the CNS. Under these circumstances there is a small but measurable transfer of viral-mediated DNA into the CNS [77]. At its current level it is unlikely that this transfer is of sufficient magnitude to be more advantageous than using the microglial population for CNS gene delivery. Undoubtedly, further work will be necessary to define a neurotropic vector capable of widespread CNS gene delivery at a safe and effective level. In view of these problems investigators are also looking at cellular grafts for CNS repopulation.

The most promising of these approaches is the exploration of neural progenitors for gene transfer and expression. A key advance has been the demonstration that human foetal tissue is capable of engrafting in the mature CNS. In Parkinson's disease foetal grafts have formed neural connections and have persisted with some clinical effect for at least 18 months [78,79]. Such experiments indicate the potential for grafting cells within the CNS, but these do not achieve the diffuse repopulation achieved with microglia and are presumably only applicable to treatment of relatively localised abnormalities. In animal studies, investigators have immortalised cells from the external granular layer of the cerebellum by transducing with cellular oncogenes such as *myc*. Following injection into the ventricular system these lines stably engraft throughout the CNS and can differentiate into both neurons and glia [80]. Such cells have been injected into the ventricles of MPS-VII mice and have formed intracranial grafts even in the absence of prior conditioning or concurrent immunosuppression; 97% of animals have shown evidence of diffuse engraftment although with some degree of regional variation. All regions had measurable enzyme activity at a level of greater than 2% of normal levels. In longer range experiments transplanted animals behaved normally, with no suggestion of the neurodegenerative changes that usually occur in MPS-VII mice [81].

Similar evidence for this approach has been provided by studies in which neural progenitor cell lines were transduced with the α subunit of the

enzyme β -hexosaminidase (β -N-acetylglactosaminidase, deficient in the LSD Tay-Sachs disease) and the marker gene β -galactosidase [82]. Transfer of the cell line into foetal and newborn mice once again confirmed the potential for long-term diffuse engraftment and detectable enzyme levels above aged-matched controls. Enzyme levels were highest when transplant was undertaken in foetal tissue.

Of course, there are many difficulties in transferring this approach into human subjects; not least are the concerns surrounding immortalisation of neural cells with known oncogenes, and the potential for secondary tumour induction. Although there have been no problems with secondary tumours in mice, this remains a significant safety issue in human trials.

The alternative approach of expanding neural progenitors without immortalisation is probably technically feasible but so far such cells have failed to engraft properly and will not be able to disseminate within the CNS. Consequently, more work will be needed before reliable methods for CNS enzyme transfer are obtained.

14.5 SUMMARY

The lysosomal storage diseases offer some difficult technical challenges in gene therapy and also offer some of the best opportunities for direct benefit. Where a disorder such as type I Gaucher's disease affects only one major cell type and can be successfully treated by allogeneic bone marrow transplant, genetic manipulation of autologous bone marrow offers a real potential for cure at lower morbidity and mortality. At the other end of the spectrum, there are many disorders that affect additional tissues, including the CNS, and in which bone marrow transplant has made little impact. These diseases will require a continuing major research investment before clinical benefits from gene therapy are seen.

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15 Prospects for Gene Therapy of HIV Infections and AIDS

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15.1 INTRODUCTION

The acquired immunodeficiency syndrome (AIDS) is characterized by immune dysfunction leading to opportunistic infections (e.g. candidiasis and *Pneumocystis carinii* pneumonia) and occasional emergence of malignancies (i.e. lymphomas and sarcomas) [1,2]. Full-blown AIDS ultimately leads to death. A hallmark of AIDS is the depletion of CD4+ T lymphocytes, which play key roles in the host immune defenses [3,4].

AIDS is etiologically linked to the human immunodeficiency virus (HIV), a member of the lentivirus subfamily in the *Retroviridae* family [5]. Over the past few years, much progress has been made in understanding viral pathogenesis and in identifying new molecular targets for clinical and genetic intervention. New and powerful chemotherapeutic combinations have been developed. For example, current findings indicate that protease inhibitors, in combination with two other anti-HIV-1 drugs with different mechanism of action, can reduce the amount of HIV-1 in 85% of treated patients to undetectable levels [6]. The long-term efficacy of drug combinations is, however, unknown, and there are hints of emergence of resistant viruses [7]. Thus, it is imperative that one does not abandon consideration of non-chemotherapeutic alternatives for combating HIV-1. Gene therapy represents one such alternative. In a non-exhaustive fashion, we review here some ideas on gene therapy for HIV-1. Because gene therapy targets different steps of HIV-1 replication, a brief synopsis of the virus life cycle is presented first.

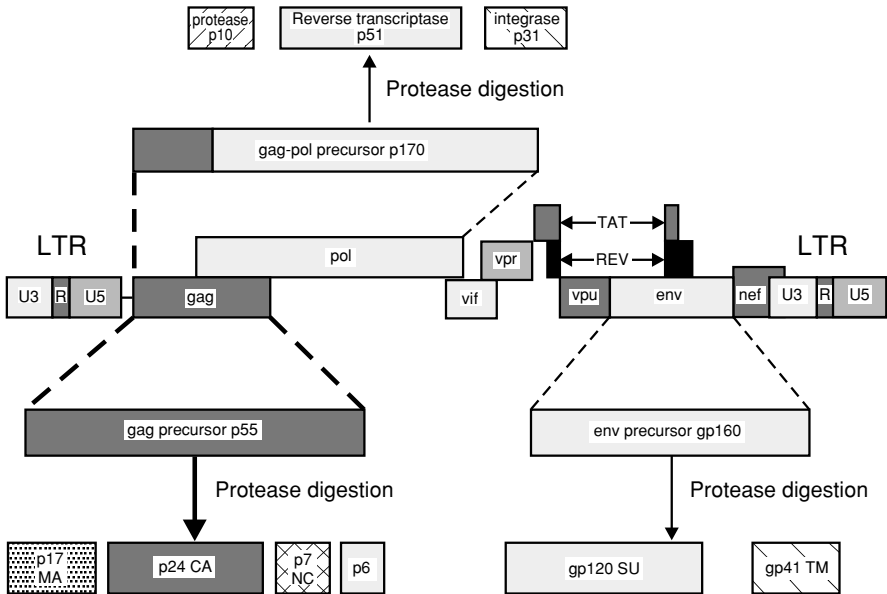


Figure 15.1. Structure of HIV-1 genome.

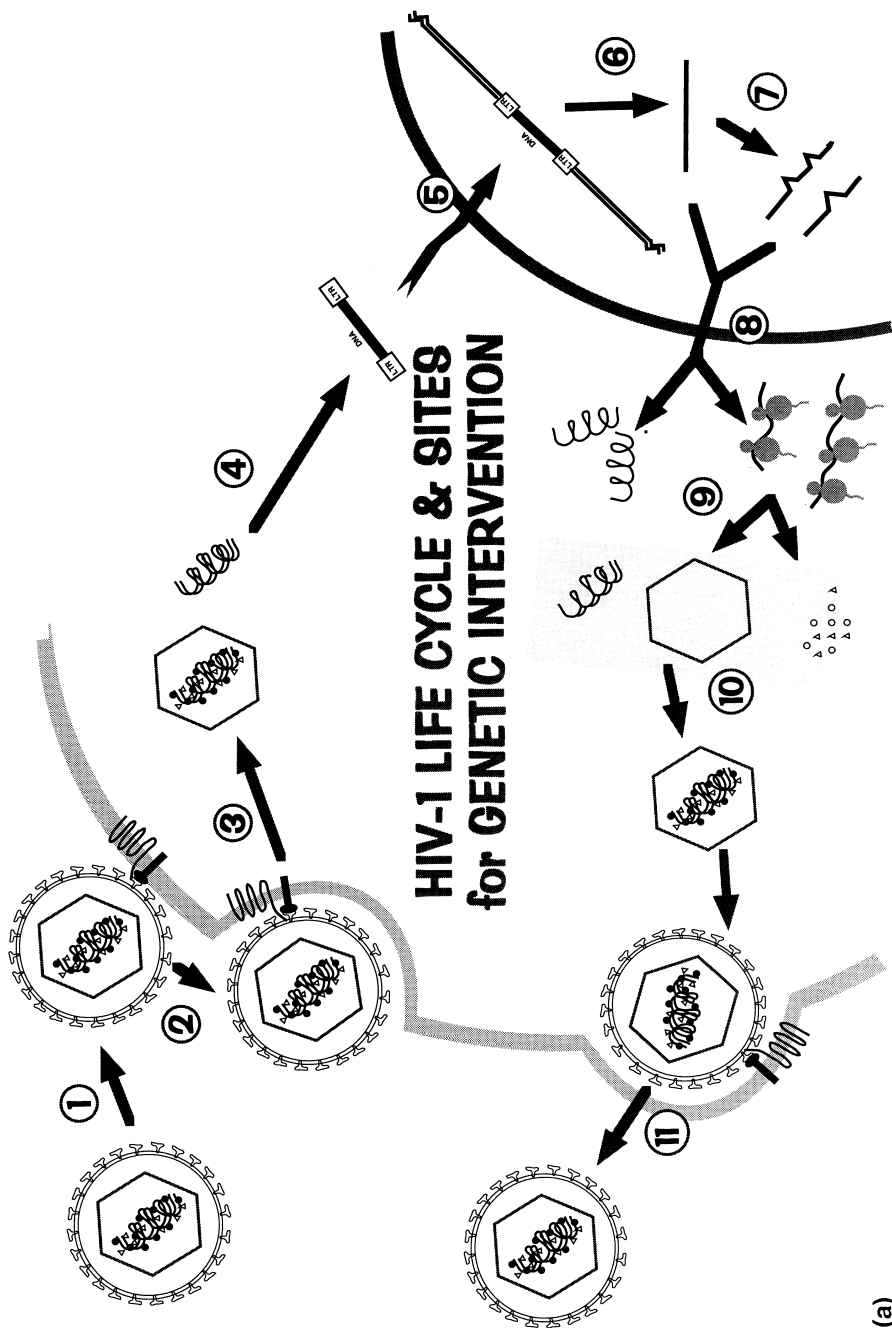
15.2 LIFE CYCLE OF HIV

Figure 15.1 shows the genomic organization of the HIV-1 provirus. The genome contains *gag*, *pol* and *env* structural genes flanked by long terminal repeats (LTR). The LTR, divided into segments termed U3, R, and U5, contains a viral promoter/enhancer as well as the polyadenylation sequences. *Gag* encodes p17 (matrix), p24 (capsid), p7 (nucleocapsid), and p6. The *pol* gene encodes the HIV-1 protease, reverse transcriptase (RT), and integrase. *Env* encodes the glycoprotein envelope of the virus, which interacts with cell surface receptor(s). *Env* is first expressed as a precursor (gp160), which is processed to the surface (gp120) and transmembrane (gp41) proteins. By contrast with simpler retroviruses, the HIV-1 genome has six additional open reading frames (ORF). These include genes for accessory proteins *nef*, *vpu*, *vif*, *vpr* (or *vpx* in HIV-2 and Simian immunodeficiency virus; SIV), and the auxiliary genes, *tat* and *rev*, which are required for virus growth. The gene products of the accessory and auxiliary genes are potential gene therapy targets.

15.2.1 RECEPTORS

Figure 15.2 illustrates schematically the HIV-1 life cycle with potential points for intervention. The virus infects cells through interactions between envelope glycoprotein and the HIV-1 receptor(s) on the surface of T lymphocytes and/or macrophage cells [8,9]. The major HIV-1 receptor is CD4 [10–12], which is normally involved in the recognition of class II major histocompatibility complex (MHC class II) of antigen presenting cells. It is proposed that the high-affinity binding of Env gp120 to CD4 exposes a co-receptor binding domain. This domain, thought to be the V3 loop of gp120, plays a critical role in determining viral tropism [13]. Upon interaction with the co-receptor, conformational changes in gp41 are triggered, releasing its N-terminal hydrophobic peptide to initiate membrane fusion.

Co-receptors for HIV-1 have been recently characterized. Different co-receptors have been identified for T-cell-tropic and macrophage-tropic isolates. For T-cell-tropic HIV-1 isolates, the co-receptor is a seven-transmembrane, G protein-coupled chemokine receptor known as fusin/LESTR/CXCR4 (leukocyte-expressed seven transmembrane-domain receptor) [14–16]. The normal ligand for fusin is the lymphocyte chemoattractant, stromal-cell-derived factor (SDF-1) [17,18]. SDF-1 belongs to the CXC chemokine family with a four-cysteine motif in which the first two cysteines are separated by one amino acid [19]. The major co-receptor for the macrophage-tropic HIV-1 is CCR5. The natural ligands for CCR5 are the β -chemokines RANTES (regulated-upon-activation, normal T expressed and secreted), MIP-1 α and MIP-1 β (macrophage inflammatory protein), members of the C-C chemokine family [20,21]. It is of considerable medical interest that some individuals who have homozygous mutations (a 32 bp deletion within the coding region of the gene) in the CCR5 [22–24] allele remain seronegative despite being repeatedly exposed to HIV-1 [25]. Epidemiological studies have estimated the allele frequency for CCR5 mutations. Lindau *et al.* [22] and Samson *et al.* [23] examined 122 of 704 uninfected individuals of Western European descent and 46 of 372 samples from Venezuelan, black and/or Japanese ancestries, respectively. In Caucasians, they found that 81–83% were homozygous for wild-type CCR5, 16–18% had one wild-type allele and one mutant allele, and 1% were homozygous for mutant CCR5. In the other racial populations, CCR5 mutations were not seen. Samson *et al.* noted that among Caucasians, no HIV-1 infected person was homozygous for mutant CCR5 and that the frequency of heterozygosity for CCR5 was lower in infected Caucasians than in the general population (10.8% versus 16%). These findings suggest that homozygous/heterozygous changes in CCR5 are fully or partially protective for HIV-1 infection. Slightly different observations were made in another study [24]. Dean *et al.* [24] analyzed 1955 infected and high-risk-uninfected individuals. They confirmed that no HIV-1-infected Caucasian was



(a)

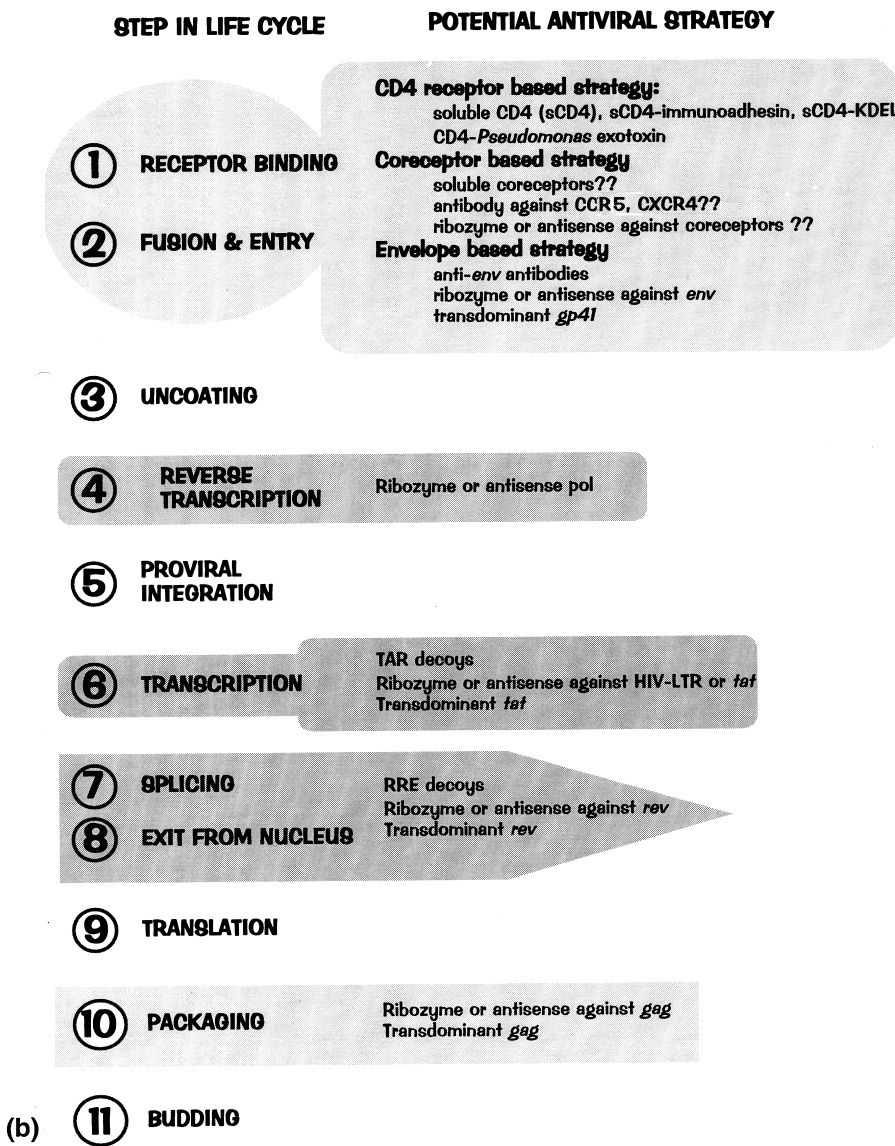


Figure 15.2.(a) (opposite page) Life cycle of HIV-1. (b) (above) Sites for genetic intervention.

homozygous for mutant CCR5 and that more (2.8% versus 1%) high-risk uninfected individuals had deletions in both CCR5 alleles compared to the general population. However, differing with Samson and colleagues, Dean *et al.* observed similar frequencies of CCR5 heterozygotes (15% versus 14%) in the infected and uninfected populations, although there were significantly more heterozygotes among 'long-term nonprogressors.' This latter finding suggests that a mutant copy of CCR5, though unable to protect against infection, might slow disease progression. Of note, homozygous mutations in CCR5 alleles do not apparently affect immune functions, implying that this chemokine receptor normally serves a redundant function.

15.2.2 INTRACELLULAR EVENTS

Entry of the virus into cells is followed by uncoating of virion RNA in the cytoplasm. The RNA genome is reverse-transcribed to generate double-stranded DNA copies [26,27]. The DNA is transported into the nucleus, where viral integrase catalyzes its insertion into the host chromosome. Chromosomal integration makes the provirus an integral part of the cell. This property of retroviruses makes eradication of the virus from the infected individual difficult.

Next, the integrated provirus is transcribed by RNA polymerase II. Initially, viral mRNA is spliced into multiple transcripts which code for viral Tat and Rev. Tat is a transcriptional transactivator of the proviral promoter. It interacts with an RNA target, Tat-responsive (TAR) element, found in the R region of the 5' LTR [28,29]. Tat facilitates the formation of transcriptionally competent complexes at the LTR promoters (see [30]). Rev is a post-transcriptional regulator which interacts with an RNA target, Rev-responsive element (RRE), in *env* [31]. Rev facilitates the transport of viral RNA from the nucleus into the cytoplasm, thereby modulating the efficiency of translation of unspliced and/or singly spliced viral mRNAs [32,33].

In the cytoplasm, viral mRNAs are translated and various structural and accessory proteins are produced. Virion assembly ensues, and viral particles bud through the cellular membrane to begin another round of infection.

15.3 STRATEGIES FOR ANTIVIRAL THERAPY

A goal of HIV-1 gene therapy is to target infected cells without adversely affecting normal cells. Optimally, approaches should be general and should be insensitive to sequence variations in different HIV-1 strains. Considering these points, we discuss several HIV-1 gene- and protein-based therapy approaches: (i) interfering with one or more steps in HIV-1 replication using nucleotide- or

protein-based antivirals, (ii) inducing death of HIV-1 infected cells, and (iii) augmentation of immune function(s) through gene transduction.

15.3.1 INTERFERENCE WITH HIV-1 REPLICATION

15.3.1.1 Interfering with Virus Entry

Binding of virus to cell surface CD4 is a first step in HIV-1 replication. Approaches to block HIV-1/CD4 interaction have been considered. Soluble CD4 (sCD4), containing only the N-terminal domain, has been found to bind avidly to gp120, blocking HIV-1 entry [34]. However, the half-life of sCD4 is too short to be clinically useful. One remedy is to link the IgG heavy chain to sCD4 to form a hybrid molecule, sCD4-IgG [35–38]. CD4-IgG has advantages, including increased half-life, greater avidity for HIV-1, ability to carry out antibody-dependent, cell-mediated immune response against HIV-1 infected cells, and ability to cross the placenta. The clinical efficacy of sCD4-IgG has yet to be demonstrated [36,39]. In fact, there are some indications that sCD4-IgG isolated from the blood of treated patients might no longer block virus entry. Shortcomings in sCD4 therapy include virus resistance [40,41] and interference with normal CD4 cellular function [42,43].

Various approaches can be developed to target HIV-1 co-receptors (see above discussion) such as CCR5. It was reported recently that a fusion protein incorporating CCR5 into a human monoclonal antibody-like protein neutralizes HIV-1 [44]. Agents such as this are potentially useful therapeutics. Analogues of chemokines [45] may also be useful inhibitors. Efforts are currently directed towards developing analogues that retain their receptor binding capabilities but lack chemotactic and leukocyte-activating properties [46].

15.3.1.2 Interfering with Transcription/Translation and/or Virus Assembly

15.3.1.2.1 Protein-Based Therapy

One basic strategy is to overexpress mutant proteins with dominant negative phenotypes. The principle is that defective mutant protein can remain competent for protein–protein interactions, thereby sequestering and potentially inhibiting wild-type function. Practical applications of this transdominant strategy have been demonstrated for the HIV-1 Tat, Rev, Gag, and Env proteins.

Many investigators have constructed transdominant negative Tat proteins. These transdominant moieties function either by forming inactive hetero-complexes with the wild-type Tat or by sequestering cellular factors involved in HIV-1 gene expression [47]. The therapeutic potential of transdominant

Tat is limited by the fact the efficacy is achieved only when the mutant Tat is expressed at large molar excess over wild-type Tat. Furthermore, Tat-independent transcription can occur [48–51]; thus, removal of Tat function may not necessarily cripple the virus.

One of the best studied dominant negative proteins for HIV-1 is a transdominant Rev mutant, RevM10. This mutant retains the ability to oligomerize, bind RRE, and localize to the nucleus. However, RevM10 is intrinsically inactive and can interfere *in trans* with the function of wild-type Rev. The inhibitory mechanism is suggested to include competition for binding of the viral nuclear transcript, and/or formation of mixed multimers [52]. RevM10 has been shown to be effective in protecting T cells against HIV-1 infection [53–55] and has been found to inhibit production of infectious HIV-1 particles in chronically infected T cells [56]. A phase I clinical trial using an expression vector encoding RevM10 delivered by gold microparticles to enriched CD4+ cells showed that the RevM10 transduced cells were more resistant to HIV-1 infection and survived better compared to cells transduced with a deletion mutant control (Δ RevM10) [57]. To further improve the antiviral effects of transdominant proteins, vectors expressing both transdominant *tat* and *rev* were constructed [58,59]. These vectors were found to be more resistant to HIV-1 infection in cultured cells.

Transdominant Gag has also been found to be effective in inhibiting HIV-1 replication, probably by interfering with multimerization and assembly of the viral core [60]. However, its usefulness is limited by difficulties encountered in achieving stable expression of this protein [61].

HIV-1 Env is another potential therapeutic target. Env is comprised of two subunits, gp120, involved in CD4 binding, and gp41, which contains the fusogenic domain. A transdominant gp41 mutant has been reported. This mutant, 41.2, has a polar substitution at the second amino acid of gp41, which disrupts the hydrophobic interaction between Env and its cell-surface receptor [62,63]. The 41.2 mutant was found to inhibit syncytia formation and virus infectivity in transient experiments using CD4+ HeLa cells.

Transdominant mutants have several limitations. Mutant forms of Tat and Rev still bind RNAs, though this means they could potentially disrupt normal cellular functions. Additionally, transdominant strategies affect only post-integration events. While this can slow virus spread, it cannot eliminate viral burden. Hence, transdominant strategies are vulnerable to the eventual selection of resistant viruses that may be more pathogenic [64,65]. Finally, mutant transdominant proteins are expected to elicit cytotoxic T cell responses, which will eliminate cells expressing these gene products. It is possible that this immune response can be evaded by tagging therapeutic genes with motifs that reduce the immunogenicity. One such motif, a glycine–alanine repeat, has been identified in an Epstein–Barr virus (EBV) nuclear antigen (EBNA-1) protein [66].

15.3.1.2.2 *Nucleic Acid Based Therapy*

Sequence-specific nucleic acids that inhibit HIV-1 function but not host gene expression can also be used for gene therapy of AIDS. RNA decoys, antisense oligonucleotides, and ribozymes are some such effectors. The effectiveness of nucleic acid based strategies depends on many factors, including the ability to co-localize target and effector molecules inside cells, and the accessibility of targets to effectors. Some of these issues have been discussed in an earlier review [67].

(A) *RNA decoys*

RNA decoys exploit one unique aspect of HIV-1 gene expression. Two key HIV-1 regulatory proteins are Tat and Rev. In order to activate gene expression, both bind to specific viral RNA sequences, TAR and RRE (see [68]). RNA decoys mimic authentic TAR and RRE RNAs but in a non-functional manner. Thus, decoys sequester viral and cellular factors that are essential for HIV-1 regulatory functions. TAR decoys have been expressed from tRNA polymerase III as a chimeric tRNA-TAR transcript [69] with variable success in human T cells (see [70]). Polymeric TAR (up to 50 tandem copies) have also been expressed under the control of HIV-1 LTR [71].

RRE decoys have also been expressed as tRNA-RRE chimeras and have been shown to have some efficacy in inhibiting HIV-1 replication [72]. One general concern with RNA decoys is the unintentional sequestration of cellular factors, since many cellular factors are known to bind TAR and/or RRE (see [73]). To address this concern, a minimum RRE decoy has been constructed that does not associate with cellular factors [74-76]. This 13-nucleotide minimum Rev binding RNA affected Rev function in transient assays and protected CEM cells from HIV-1 infection [77,78]. Selection for RRE decoy expression in bone marrow or cord blood CD4+ hematopoietic progenitor cells also protected these cells from HIV-1 infection [79].

(B) *Antisense*

Antisense molecules may work by blocking translation and/or promoting mRNA degradation by RNase H [80]. These molecules can be delivered as nuclease-resistant chemically modified analogues (e.g. phosphorothioate [81-83] or methylphosphate [84] analogues) or be expressed intracellularly using a eukaryotic promoter [85,86]. Antisense molecules have been made to many HIV-1 sequences, including TAR [85,87-90], U5 [85,87-91], and the primer-binding site [85,87-91]. Antisense has also been targeted to *tat* [89,92-94], *rev* [89,95], *pol* [95,96], *vpu* [86], *gag* [95,97], and *env* [98]. Most intracellular antisense genes are delivered either via retroviral [86] or

adeno-associated viral (AAV) [85] vectors. Recently, a novel expression method in which the antisense sequence is inserted into a tRNA(Pro) backbone has been described [92]. One principle in antisense therapy is that the approach is more effective if several different sites are targeted simultaneously [85,87,89,91,95].

A modified antisense molecule that utilizes RNase L, a ribonuclease involved in the action of interferon, for scission has been proposed by Silverman's group [99–105]. In this method, antisense oligodeoxyribonucleotides are covalently linked to 5' phosphorylated 2'-5'-linked oligoadenylates (also known as 2-5A). These modified 2-5A antisense oligonucleotides anneal specifically to target RNA sequences, whereupon RNase L is activated to cleave the target RNA. RNase L is an enzyme ubiquitously found in many mammalian, reptilian, and avian cells.

(C) *Ribozyme therapy*

Ribozymes are antisense RNAs that can catalytically cleave substrate RNAs. Since this reaction does not consume the ribozyme, a single molecule of ribozyme is potentially able to process many substrate molecules, theoretically allowing ribozymes to be effective at lower concentration than conventional antisense oligonucleotides. The core ribozyme motif(s) essential for activity have been characterized [106,107]. Hammerhead [106] and hairpin [108] ribozymes have been designed to inactivate HIV-1 RNAs.

A minimal hammerhead ribozyme is composed of three helices and a catalytic core (Figure 15.3). This ribozyme cleaves target RNA containing a GUX consensus (X can be C, U, or A) to generate 2',3' cyclic phosphate and 5' hydroxy termini [107]. The hammerhead ribozyme can discriminate between substrates with single base substitutions in the recognition site [109] as well as closely related RNA [110]. The cleavage efficacy of ribozymes can be improved by the presence of RNA-binding proteins such as HIV-1 p7 nucleocapsid protein, which accelerates the association/dissociation of RNA helices [111,112].

Hairpin ribozymes are modeled after the minus strand of the satellite RNA of tobacco ringspot virus (sTobRv) [113]. This ribozyme has four helices separated by two internal loops (Figure 15.4). Substrate anneals to the ribozyme at helices 1 and 2. Cleavage occurs at the 5' end of a guanosine within the conserved cleavage site located in loop A of the substrate. The sequence requirements in the target RNA are relatively simple: BNGUC where B can be U, C, or G but not A, and N can be any nucleotide [114].

To date, HIV-1 ribozymes have targeted against *gag* [115], *env* [116,117], *pol* [117–119], *tat* [120–122], RRE [117], LTR [120,123,124] R region [117,125], and U5 region [117,126–128]. Ribozymes simultaneously targeted to multiple sites have also been described [116,129].

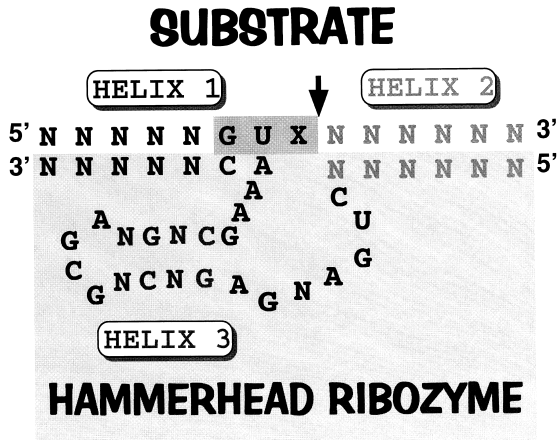


Figure 15.3. Consensus structure of the hammerhead ribozyme.

(D) *DNA vaccines*

Recently, it was shown that direct injection of DNA into hosts can generate protective immunity. For influenza virus [130], a vaccine was delivered by direct injection of plasmid DNA encoding the viral proteins and/or genes encoding immunostimulatory molecules such as the B7/BB1 molecule [131] to induce antigen-specific cytotoxic T lymphocytes. For HIV-1, direct injection of gp160-expressing plasmid has generated neutralizing antibodies [132].

15.3.2 INDUCTION OF CELL DEATH

Even when retroviral RNA is completely eliminated from the host, integrated proviral DNA persists. In order to clear HIV-1 virus from the body, strategies that induce cell death can be contemplated. Selective induction of cell death serves to eliminate HIV-1 infected cells and integrated HIV-1 provirus. One strategy uses CD4 as a targeting agent to deliver cytotoxic molecules to HIV-1 infected cells. Infected cells, expressing surface gp120, can be targeted by CD4 either as a protein conjugate (e.g. ricin) [133] or a fusion construct (e.g. *Pseudomonas* exotoxin conjugate) [134,135]. Indeed, it was found that CD4-*Pseudomonas* exotoxin was extremely potent in selectively ablating chronically infected cells (IC_{50} of less than 1 nM) and controlling HIV-1 spread [134,136]. One limitation to CD4 targeting is the requirement for cell-surface gp120, which occurs only during a productive infection.

The HIV-1 LTR can be used as an inducible promoter for toxic genes. Theoretically the LTR is silent until activated by the viral Tat protein. Infec-

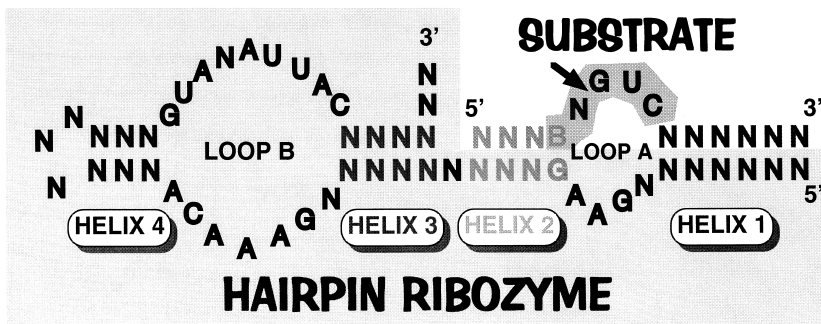


Figure 15.4. Consensus structure of the hairpin ribozyme.

tion of cell by HIV-1 results in Tat synthesis triggering the LTR, thereby initiating transcription of the toxin cDNA. The HIV-1 LTR has been used to express the poliovirus protein 2A [137] and the diphtheria toxin A (DT-A) chain genes [138–142].

One shortcoming with HIV-1 LTR is that its Tat-independent basal activity is not null [48,49]. In a setting where the linked agent is extremely toxic (i.e. a single molecule of DT-A will kill the cell [143]), leaky promoters are not usable. There are attempts to modify the LTR to include RRE sequences as a further level of control to reduce basal expression [139,141,142].

15.3.3 IMMUNIZATION AND AUGMENTATION OF IMMUNE FUNCTION

The global solution to AIDS requires the development of an effective vaccine. There are many vaccine strategies. From a gene therapy perspective, we will limit our discussion to the concept of a live attenuated virus vector.

One concern about using a live attenuated virus vaccine is the risk that the crippled virus will revert to virulence. For HIV-1, it has been proposed that a *nef*-deleted replication-competent lentivirus could be used as a vaccine. Deletion of the *nef* gene from SIV rendered the virus non-pathogenic in immunocompetent adult rhesus macaques [144, 145] though not in newborn animals [146]. Inoculation with attenuated, *nef*-defective SIV was found to protect macaques against subsequent challenges with pathogenic SIV [147,148].

Live attenuated retroviruses permanently integrate into the host-cell chromosome. This fact raises concern about safety and the possibility of eventual reversion to virulence. It is desirable to endow live attenuated viruses with additional safety features through gain-of-function. Indeed, the use of herpes simplex virus thymidine kinase (HSV-tk) gene as a condi-

tional gained-function for HIV-1 vectors has been proposed [149]. Cells expressing HSV-tk can be killed efficiently with ganciclovir. A limitation of the gain-of-function safety strategy is a lack of assured selective pressure to prevent loss of the suicide feature from the viral genome. Strategies that preserve gained 'safety' elements in a live attenuated viral genome need to be developed.

Two other approaches in immuno-gene therapy merit discussion. The first is the intracellular expression of antibodies. It has been reported that expressing the heavy and light chains of the Fab fragment of the monoclonal antibody directed against the conserved CD4 binding region on gp120 [150–153] in cells resulted in antibodies which protected lymphocytes from HIV-1 infection [154–157]. The second is the augmentation of the immune system through modulation by lymphokines/cytokines. Interferons (IFN) are potent anti-viral molecules that have biological properties which can affect various steps of the HIV-1 life cycle, ranging from penetration to particle maturation [158]. IFN α , β , and γ have been shown to inhibit HIV-1 replication in cells [159–163]. Genetic approaches in which IFN α , β and γ that is expressed from HIV-1-LTR have been made, rendering these molecules TAT/HIV-1 inducible. These TAT-inducible IFNs were found to be effective in controlling HIV-1 infection in cultured T cells [164], primary lymphocytes, and in cultured T cells that had been implanted into SCID mice in a humanized SCID model system (see [165]).

15.4 DELIVERY AND SELECTION STRATEGIES FOR GENETIC ANTIVIRAL INTERVENTION

Practical issues in successful gene therapy include optimal delivery and maintenance of expression. Delivery modalities may be either viral or non-viral. The most widely studied system for gene delivery uses murine retroviral vectors [166–168]. These are relatively safe and efficient. However, their propensity to integrate randomly into the host genome presents theoretical risks for insertional mutagenesis. Safer vectors include self-inactivating E-vectors [169] and cre-lox based self-deleting retroviruses [170]. In self-inactivating E vectors, large direct repeats, flanking the encapsidation (E) signal, are exploited to remove the E which is necessary for efficient packaging of viral RNA into virions. The direct repeats can be constructed from parts of the selectable marker to insure E is deleted from all integrated provirus upon application of selection pressure. The cre-lox based vectors have the ability to excise themselves after inserting the gene of interest into the genome. In this vector, the gene of interest or a gene expressing a selectable marker containing a *loxP* sequence at one end (away from the R region) is cloned into the U3 region of the retroviral vector while the *cre* recombinase gene is cloned

between the LTRs. During reverse transcription, duplication of regions within the LTR occurs, generating two *loxP* sites and enclosing the vector sequences and the *cre* recombinase within these sites. This will then allow *cre* recombinase to excise, from the provirus, sequences unrelated to the transcription of the U3 gene, including viral sequences and the *cre* recombinase itself. Such a strategy has the advantage that, besides removing viral sequences, *cre* recombinase is permitted to express only transiently, reducing immune response against it and minimizing risk of translocation in cells carrying more than one *loxP* site. Murine retrovirus approaches are also limited by the requirement for dividing cells. In contrast, HIV-1-based lentivirus vectors [171,172] have the ability to integrate into terminally differentiated cells like the monocytes/macrophages or the glial cells. Other vectors that can work in non-dividing cells include defective herpes simplex viral (HSV) vectors [173] as well as AAV vectors [157]. Unfortunately, gene expression from HSV vectors was found to be transient [173].

AAV vectors have also been useful for delivery [85]. AAV are naturally defective, single-stranded DNA viruses which require co-infection with helper viruses (usually adenovirus, herpesvirus, or vaccinia virus) to replicate. AAV-based vectors [174] are simple since they only require the viral inverted terminal repeats (ITR) (145 nucleotides each) to flank the gene of interest. The viral genes *rep*, encoding genes involved in replication and integration, and *cap*, encoding structural genes, can be supplied *in trans*. The major constraint with AAV is that only 4.7 kb of foreign DNA can be accommodated. Potential advantages of this system include its ability to infect non-dividing cells and its stable expression. Moreover, since AAV may integrate preferentially to a single site in chromosome 19 [175], the risk of insertional mutagenesis is minimized. In practice, AAV vectors carrying foreign genes integrate poorly and probably non-specifically.

Non-viral modes for gene delivery into T cells have also been considered. One approach uses gold particles [57,176]. The DNA is coated onto metallic/gold particles which are introduced into cells/tissues using a 'gene gun.' Another approach is liposome-mediated delivery. In this method, polycationic lipids are mixed with plasmid DNA, forming liposomes which will then fuse with the target cell and mediate gene transfer [177]. Different formulations of lipids have been developed. These usually consist of mixtures of a neutral co-lipid, e.g. DOPE, with a cationic lipid or cytofectin to form cationic liposomes, which are then mixed with DNA before being introduced into cells. Compared to viral vectors, this method of introducing DNA into cells is relatively inefficient [178].

Maintenance of the transferred gene can be achieved either through integration or through preservation as an extrachromosomal element. Retroviral and adeno-associated viruses may result in integration. The ability to use episomes would be advantageous for certain purposes. EBV [179,180] and

BK virus vectors [179] can maintain high levels of expression as episomal elements. EBV episomes have been found to replicate in lymphoid cells at ~10–50 copies per cell, while BK virus can replicate in diverse cell types to ~150 copies per cell.

To insure long-term expression, an appropriate selectable marker is essential. Although neo-resistance due to expression of a neomycin phosphotransferase DNA has been popularly used *in vitro*, its applicability *in vivo* is limited [181]. A promising marker useful for gene therapy is the multidrug resistance gene (MDR-1) (see [182,183]). MDR-1 encodes a 170 kDa plasma membrane P-glycoprotein in the ATP-binding cassette (ABC) family of transporters. Surface expression of MDR-1 allows for easy detection and selection of cells containing this transgene. Mutations in different regions of the MDR-1 gene change the relative resistance to the different drugs, making it possible to construct 'designer' MDR-1 gene that distinguishes cells containing the transgene from the endogenous gene. Empirically, MDR-1 has been successful as a dominant selectable marker for the coexpression of many genes (see [184]), including HSV-tk [185, 186], glucocerebrosidase [187–189], α -galactosidase [190], and a subunit of the phox flavocytochrome b_{558} /gp91^{phox} [191].

As highlighted in this chapter, several potential strategies for the treatment of AIDS have been investigated and many more are being tested. To date, none of these approaches is curative. Combination strategies may be necessary to eliminate the HIV-1 virus completely. Strategies need to be developed to rationally sieve through all the options to determine which of these candidate approaches or their combinations would be the most efficacious. Furthermore, the timing for implementing therapy, as well as the identification of the most appropriate cell types to deliver the antiviral, must also be carefully considered. Small animal models like the SCID-Hu mice (see [192]) or transgenic mice carrying either the T-cell-tropic or macrophage-tropic co-receptors for HIV-1 entry would be useful for screening the many therapeutic schemes and identifying the most promising antiviral approach. Before embarking into human trials, a large primate model would be the next logical step to determine the effectiveness of the anti-HIV-1 therapy. A chimeric human and simian immunodeficiency virus (HIV/SIV chimera) has been constructed that will infect macaque monkeys [193–195]. Such a chimeric virus may be useful in assessing the effectiveness of the various anti-HIV-1 strategies in primate models that are not infectible by HIV-1. In a human clinical trial setting, identical twins discordant for HIV-1 infection [196] would be the most useful, since T cells from the uninfected twin can be 'protected' and the effect of this protection can be studied *in vivo*. Hence, while gene therapy holds great promise for the treatment of AIDS, many uncertainties still remain.

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16 The Development of the Regulatory Process in Europe for Biological Medicines: How it Affects Gene Therapy Products

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16.1 REGULATION OF BIOLOGICS IN EUROPE

16.1.1 HISTORICAL INTRODUCTION

Modern concepts of quality assurance and safety of biologics have their origins in the 19th century, with the beginnings of antisera production in Europe. The initial appearance of biological medicines, or biologics as we know them, began in the late 19th century with the development of various antisera for the treatment of infectious diseases, such as diphtheria. In Germany, Erich Wernicke and Emil von Behring had pioneered the development of an antiserum to diphtheria toxin, which was raised by immunising horses with the toxin, and this antiserum had shown promising results in the treatment of diphtheria [1,2]. In collaboration with the chemical company Farbwerke Meister, Lucius und Brüning Höchst, the predecessor of the well-known Hoechst AG, a daily production of 1000 vials of antiserum was being achieved (Figure 16.1). However, the procedure for making the antiserum was not protected by patents, and Behring worried that his competitors would badly imitate this procedure and manufacture an inferior, less potent, antiserum. This might, he felt, eventually discredit his therapy rationale. He also knew that the pharmacists who dispensed the antiserum would not, because of the complicated and cumbersome testing methods, be able to assure the antiserum's potency and safety. They would not be able to distinguish a 'good batch' of antiserum from a 'bad batch'. Thus, pressure mounted for the



Figure 16.1. Serum and toxin preparations individually signed by Behring.

establishment of central state facilities with responsibility for testing those medicines for which pharmacists did not have the necessary capabilities.

On 3 November 1894 a meeting of the heads of the health authorities of the German Empire was convened at the Kaiserliches Gesundheitsamt, the then recently founded premises of the National Health Authority in Berlin, to discuss a proposal for establishing specific regulations on a 'novel therapy' for the treatment of diphtheria. It was Behring who, with the assistance of such illustrious scientists as Robert Koch and Paul Ehrlich, had initiated the meeting and who presented the proposal. In Behring's own words:

If it should come to pass that the state executes a control over diphtheria antiserum, this control should have two directions: firstly, with reference to appropriate manufacturing practices and, secondly, with reference to the finished drug product. On the basis of my experience, I am convinced that above all the finished drug product, before it comes to distribution, requires controls by the state. On the other hand, if this control is performed adequately, it should suffice to allay the need for any other control activity.

This was the main topic considered at this meeting and by its end the following resolutions had been adopted:

Diphtheria antiserum shall contain not less than 100 immunising units per ml. The serum shall not contain noxious substances. The possibility of transmission of zoonotic diseases to humans by the antiserum shall be excluded. The serum must not contain preservatives in hazardous quantities.

The objective of these resolutions was to guarantee an effective immunising potency for each batch of antiserum and to assure its safety. However, the means for accurately quantifying the potency of diphtheria antiserum was not available until about three years later, with Paul Ehrlich's pioneering work in the field of biological standardisation. In 1897, he established a biological unit for diphtheria antiserum, which was defined as a portion of the content of a particular 'standard' preparation of dried antitoxin [3]. This was an extremely important development that yielded the principles of biological standardisation for the now many different biologics that are currently manufactured. Ehrlich's laboratory-based investigations strongly indicated that use of a single standard preparation worldwide for calibration of assays permitted direct comparison of results obtained in different laboratories at different times and produced using a spectrum of assays, formats or methods. The Ehrlich standard for diphtheria toxin was distributed widely and, despite problems encountered during World War I, its unitage was maintained by the use of similarly calibrated secondary standards. In 1922, the League of Nations adopted the Ehrlich unit as the International Unit, thus instigating the concept of a worldwide status for standard preparations and their associated unitage. This activity has been maintained (later by the World Health Organization (WHO) of the United Nations, which succeeded the League of Nations) to the present.

An equally impressive historical example of a standard for a biologic is that for insulin, introduced by Sir Henry Dale, a British scientist, in 1923. Before then, attempts had been made to measure the potency of insulin and, indeed, of biologics in general in 'units' which were defined in terms of actions in laboratory animals. The unit of insulin was initially proposed as the dose which would throw two rabbits out of three into convulsions or, according to a further proposal, which would produce hypoglycaemic convulsion in six out of twelve mice. Such futile approaches were overcome by Sir Henry Dale in 1923 at a conference in Copenhagen. He presented a highly stable preparation of freeze-dried insulin and made clear that the only sensible way forward was to define the unit of insulin in terms of a certain amount of this reference preparation, leaving the methods of its determination to be the subject of experimental improvement [4,5]. The first international standard for insulin and the international unitage (IU) of biologic activity were established in 1926. In 1998, over 70 years later, manufacturers,

national control laboratories, physicians and patients still exclusively use the same IU. The field of biological standardisation has thus contributed much towards assuring the quality, efficacy and ultimately the safety of biologics.

In addition to Behring's resolutions, provisions were agreed to give the state the authority to inspect the manufacturer's facilities and animal herds and, prior to release to pharmacists, to examine each batch of product. Thus, the way was paved towards defining a new class of drugs or medicines, the biologics, which require novel biological tests for quality assurance and for which it was deemed necessary to have state-controlled testing capability leading to batch release. The recommendations made by the assembly in Berlin were subsequently adopted by all health authorities within the German Empire, and, from there, via the League of Nation's provisional health committee, found their way into the health regulations of most other countries.

16.1.2 CLASSIFICATION AND CHARACTERISATION OF BIOLOGICS

By the end of the 19th century, Behring's diphtheria antiserum therapy was being widely applied throughout the world. In the course of time, an increasing number of similar products and vaccines entered into clinical usage. These constituted an ever-increasing category of biological medicines, now formally classified as biologics. In the USA, the EU and most other countries today, biologics include 'vaccines, sera, anti-toxins and blood products'. In 1992, WHO proposed the following three specific features that would characterise biologics [6] and distinguish them from other types of medicines:

1. The biological nature of source materials.
2. The biological nature of the manufacturing process.
3. The need for biological methods to determine the purity and potency of the product.

Besides vaccines, sera, blood products and anti-toxins, the WHO criteria would also classify cytokines, large polypeptide growth factors, certain hormones, blood clotting factors, monoclonal antibodies and other analogous protein products, generally now collectively designated as 'biotechnology medicines', as biologics. These can be manufactured by various biotechnological processes, including recombinant gene technology, large-scale mammalian cell culture, and expression in transgenic animals. In most countries and in the EU, because of their special biological properties, they are usually subjected to similar quality assurance (QA) requirements and licensing procedures that are applicable to 'conventional' biologics such as antisera. However, some medicinal products, e.g. vitamins, antibiotics and

steroid hormones, which originally fell within the WHO definition of biologics, are no longer considered as such since biological testing has been replaced by chemical methods.

16.1.3 THE EMERGENCE OF GENETIC MEDICINES: ARE THEY BIOLOGICS?

It is within the context of the QA of biologics that new classes of medicinal agents containing genetic materials are emerging for consideration. At present, it could be conceived that there are at least three distinct categories of genetic medicines that contain nucleic acid as the active principle:

1. Nucleic acids (NA)
2. Vectors
3. Cellular vehicles

Category 1 may be subdivided into:

1. Antisense polynucleotides
2. Ribozymes
3. Plasmid DNA

Antisense polynucleotides are mainly designed to bind to certain mRNA or promoter regions of certain genes to suppress translation or transcription, respectively. They may also act as decoys for transcription factors [7]. Ribozymes are polynucleotides that catalytically cleave at certain base combinations in mRNA, and thus inhibit translation [7]. Naked plasmid DNA is usually much larger than antisense and ribozyme polynucleotides, and normally will contain gene-size constructs. As such, it will require the assistance of a device, such as a 'gene gun', or a permeating method, such as electroporation, to deliver the DNA into target cells. Naked plasmid DNA has been most commonly used for NA vaccine strategies [8].

In contrast, vectors (Category 2) are entities of NA plus materials of non-viral or viral origin, most with in-built tropisms or targeting moieties [9]. They can be subdivided into (i) non-viral complexed or condensed NA, which include simple lipid-NA combinations (DNA-liposomes) to much more complex multimolecular entities, such as minichromosomes, and (ii) viral vectors. The latter include a growing range of mostly replication-deficient viruses, e.g. retroviruses, adenoviruses, parvoviruses and arboviruses, carrying transgenes, but also might include live attenuated viruses, such as herpesviruses [10].

Cellular vehicles (Category 3) are genetically modified somatic cells of autologous, allogeneic and xenogeneic origin, which carry either normal

genes that are expressed to correct a genetic defect, or genes (human, animal, foreign) that are expressed to produce functional gene products intended to act against acquired diseases such as cancer, arthritis, AIDS etc. Genetic modification is performed *ex vivo* by the application of particular vectors [9,10]. It should be noted that, in these cases, the genetically modified cells, which are put back into patients, constitute the genetic medicine and not the vectors, which here are the tools for genetic transduction. However, cellular vehicles can also act as the means for delivery of vectors, e.g. murine cell line producing replication-deficient retrovirus particles *in situ* [11]. As mentioned, genetically modified cells are put into patients and this can be achieved by a variety of means, including inoculation, infusion, engraftment and a number of devices for implantation. For example, cells may be grown *in vitro* in collagen matrices to produce 'organoids' or 'neo-organs', or encapsulated before being introduced into recipients so as to avoid immune attack.

The range of genetic medicines is summarised in Table 16.1. Not all of these, however, are considered as 'gene therapy products'. Antisense polynucleotides and ribozymes are normally chemically synthesised [7] and their quality aspects of production will thus differ significantly from biologics. They are therefore usually excluded from consideration as gene therapy products, except when they are contained within vector NA constructs and expressed intracellularly. Vaccines comprised of naked DNA and vectorised vaccines designed to immunise against infectious agents may also be excluded, not on the basis of dissimilarity of materials but on the basis of their intended use [8]. Such vaccines will generally be given to young healthy humans. In contrast, gene therapy products are used for the therapeutic treatment of a wide variety of diseases, which afflict both young and old. Here efficacy will depend on the site and level of transgene expression and on the functional activity of the encoded protein or other molecule [9]. Integration of the transgene into nuclear DNA will often be sought. On the other hand, the efficacy of NA vaccines is measured in terms of a protective immune response; site and level of transgene expression and function of the encoded antigen may not be directly relevant to efficacy. Integration of vaccine NA into nuclear DNA will be undesirable [12,13]. Since NA vaccines will be targeted to mainly young, healthy populations, their safety will be a paramount consideration and will possibly exceed the safety requirements set for many gene therapy products, which are mostly administered to patients with life-threatening medical conditions [14].

Despite the wide spectrum of materials and agents used for their preparation, most health and regulatory authorities have considered that gene therapy products, should, because of their biological features, be considered together, or at least under a common 'umbrella' heading, for the purposes of deciding QA and safety criteria. This is perhaps because, despite structural and biological differences, gene therapy products are (almost) all prepared

from biologically sourced materials, have intrinsic biological features, contain DNA/RNA with well-characterised biological function, and require biological testing for complete characterisation. Most authorities have therefore deemed it reasonable to apply the principles of quality and safety testing that have proved effective for biologics. In particular, the principles of quality and safety testing of biologics deriving from recombinant DNA technology, which is also the enabling technology for NA/gene manufacture, have been adopted (or adapted) for gene therapy products in many cases. However, while there are certain aspects in common with conventional biologics and biotechnology medicines, there are other aspects of gene therapy products that require special attention or emphasis for QA and safety of products. These include:

1. Molecular genetics – isolation and characterisation of the transgene and any additional nucleotide sequences required for transgene expression.
2. Production procedures required to amplify transgenes and other nucleotide sequences to ensure high copying fidelity and genetic stability. There are concerns about mutations in viral vector genomes, particularly in the contained transgene.
3. Chemistry/biochemistry of complexing/condensing agents together with full characterisation of complexed DNA products.
4. Manufacture, characterisation and safety of viral vectors. There are concerns that genetic recombination will allow viral vectors that have been 'engineered' to be replication deficient to become replication competent. In addition, there are unpredictable effects of random integration of vector NA into nuclear DNA.
5. Manufacture, characterisation and safety of genetically modified somatic cells. There are concerns about possible changes in cell behaviour (e.g. heightened immunogenicity or tumorigenicity).
6. The possibility of the transgene getting into the germline.

16.2 GUIDANCE FOR MANUFACTURERS AND REGULATORY PROCEDURES APPLYING TO BIOLOGICAL MEDICINES IN EUROPE

As described in the previous sections, there is an infinite variety of biological medicines, including conventional biologics (vaccines, antisera, anti-toxins), biotechnology medicines, (cytokines, hormones, monoclonal antibodies) and genetic medicines (NA vaccines, viral vectors, genetically modified cells), that all require the formulation of regulatory frameworks to guide manufacturers seeking marketing authorisation for their products. Within Europe, the health authorities of individual countries have made their own resolutions,

Table 16.1 The range of genetic medicines

Type	Characteristics and uses
1. Nucleic acids	
Antisense polynucleotides or DNA	Short complementary sequences to mRNA or DNA. Block translation/transcription
Ribozymes	Short RNA molecules. Cleave mRNA at specific sites, block translation
Naked plasmid DNA	Contain gene length sequences, <i>in vitro</i> transfections or DNA vaccines
2. Vectors	
(i) Non-viral	
DNA (compact) in:	
Cationic liposomes	All potentially useful for <i>in vitro</i> transfections; some, e.g. cationic liposomes, useful also for <i>in vivo</i> transfections
Anionic liposomes	
Polymers	
Polymers plus adenovirus capsids	
Peptides	
Polymer particles	
(ii) Viral	
Animal pathogens	
Retroviruses (MLV, lentiviruses)	All shown to be capable of efficient transfer of 'foreign DNA' <i>in vitro</i> , but <i>in vivo</i> uses restricted to certain classes of viral vectors because of safety issues
Poxviruses (fowlpox, canarypox)	
SV40	
Mild human pathogens	
Adenovirus	
Adeno-associated virus	
Vaccinia	
Seriously pathogenic human viruses	
Alphaviruses (+ve strand RNA)	
Poliovirus	
Herpes virus	
HIV (lentivirus)	
3. Cellular vehicles	
Autologous normal cells	
Stem cells	Used primarily to replace deficient genes (e.g. in ADA, Hunter's syndrome), but also for cancer 'vaccines' (see below)
Lymphocytes	
Fibroblasts	
Hepatocytes	
Allogeneic normal cells	
Donor peripheral blood lymphocytes	For example, transduced with HSV-thymidine kinase (tk) that activates the prodrug ganciclovir for control of graft versus host disease in allogeneic bone marrow transplant recipients for leukaemias, lymphomas and myelomas

Table 16.1 (*cont.*)

Type	Characteristics and uses
Autologous or allogeneic tumour cells	Irradiated or non-irradiated cells from a wide variety of cancers have been used, either transduced by tumour suppresser genes, or by cytokine genes, or by genes for immunodulating surface molecules, to act as cancer 'vaccines' to induce or enhance patient's antitumour responses
Xenogenic cells	Murine packaging cell lines, e.g. producing retroviral particles carrying HSV-tk suicide gene, for intratumoural treatment of glioblastoma and melanoma Hamster (BHK-derived) cell lines, e.g. engineered to secrete ciliary neurotrophic factor, for treatment of amyotrophic lateral sclerosis by means of intrathecal implantation of a polymer encapsulated, retrievable device

recommendations and legislation regarding the marketing of such products. Certainly this was the case before the formation of the EU (formerly the European Economic Community, EEC), which has now grown to include 15 countries. Over a period of about 30 years since its inception, the European Commission (EC), with its headquarters in Brussels, has sought to provide guidance to manufacturers that enabled them to apply for marketing authorisation within and throughout the entire EU. From 1995, this operation involving the drawing up of guidelines, moved to the new European Medicines Evaluation Agency (EMA), situated in London's Docklands. The EMA is the administrative body of the EC handling applications for marketing authorisations and coordinating scientific advice from experts of the individual Member States, who collectively constitute the Committee for Proprietary Medicinal Products (CPMP). The Ad Hoc Working Party on Biotechnology/Pharmacy (Biotechnology Working Party from 1995) was set up by the EC in February 1986 to assist the CPMP in its activities. During the 12 years of its existence this Working Party, which includes experts from each of the Member States of the EU, has advised the CPMP in evaluating the quality aspects of the applications for marketing authorisation for medicinal products derived from biotechnology. The development of a harmonised European approach to QA, biological standardisation and control issues for biological medicines has been pursued through collaborative studies and the formulation of specific guidelines on data requirements for conventional biologics and biotechnology medicines [15].

One of the main activities of the CPMP Biotechnology Working Party has been the preparation of CPMP guidelines for the production and quality control of a range of biological medicines. More than a dozen such guidelines have been produced, including:

- Production and quality control of medicinal products derived from recombinant DNA technology.
- Production and quality control of monoclonal antibodies of murine origin.
- Preclinical biological safety testing of medicinal products derived from biotechnology.
- Production and quality control of human monoclonal antibodies.
- Radio pharmaceuticals based on monoclonal antibodies.
- Validation of virus removal and inactivation procedures.
- Harmonisation of requirements for influenza vaccine.
- Medicinal products derived from human blood and plasma.
- Minimising the risk of transmission from animals to humans of agents causing spongiform encephalopathies via medicinal products.
- Production and quality control of cytokine products derived by biotechnological processes.

Biologics, including biotechnology medicines, have one or more of the following features in common, which are important when considering QA strategies:

1. Inherent variability of composition, stability and potency.
2. They are subject to the variability inherent in biological test methods. (There may be an initial lack of an agreed system for potency measurement.)
3. Materials and manufacturing processes are susceptible to contamination by microorganisms, and/or microbial products (e.g. LPS), and/or nucleic acids (DNA, RNA).

The goal is therefore to ensure that batches of product are manufactured under good manufacturing practice (GMP) conditions with appropriate, validated, in-process controls. The final product should be well-characterised in terms of purity and activity. It should be consistent in composition, exhibit long-term physicochemical and biological stability, be free of adventitious microorganisms, especially viruses, and not contain harmful impurities or contaminants, e.g. LPS, DNA. It is the responsibility of manufacturers to provide a detailed description of the manufacturing process together with data that confirms the capacity of the process to yield the product with the desired specifications.

With regard to gene therapy products, the Biotechnology Working Party has considered that marketing authorisations for such products fall under

the scope of part A of the Annex to Council Regulations 2309/93 [16], which covers medicinal products developed by means of one of the following processes: recombinant DNA technology, and controlled expression of genes coding for biologically active proteins in prokaryotes and eukaryotes, including transformed mammalian cells. Although no marketing authorisation application has yet been submitted to the EMEA, the Working Party has drafted a CPMP guideline, which was finalised in 1994 [17]. This guideline is currently under review and changes will be made in the light of new information that has accrued in the rapidly developing field of gene therapy. In addition, an Annex to this guideline – Guidance on Safety Studies for Gene Therapy Products – has been drafted and is close to finalisation. Other relevant EC requirements include a Council Directive relating to Good Clinical Practice for trials on medicinal products (which is still being considered in draft) and Directives regulating the contained use of genetically modified organisms (90/219/EEC) [18] and the deliberate release into the environment of genetically modified organisms (90/220/EEC) [19].

The Directives and Guidelines form the regulatory framework for manufacturers applying for marketing authorisation through the centralised procedure (see below) within the EU and for the CPMP to critically evaluate any of the many different types of biological medicines that are submitted. The centralised procedure, which means applications are made directly to EMEA, leading to the granting of a European marketing authorisation by the EC, is compulsory for products derived from biotechnology and optional for other innovative medicinal products [20].

The centralised procedure, in operation from 1 September 1995, is for manufacturers with fully developed products, i.e. those which have been evaluated in phase I, II and III trials. Once an application has been made to EMEA, the CPMP nominates two of the Member State representatives as rapporteur and co-rapporteur to evaluate the application, using the resources of their own national regulatory agencies [20]. Their assessment reports are then reviewed by CPMP subcommittees, such as the Biotechnology Working Party, and their recommendations and advice considered by CPMP. Thus, all Member States are involved in this procedure, via their CPMP representatives and scientific experts serving on CPMP subcommittees. Any queries are sent back to the applicant by the CPMP. When the CPMP is satisfied a product is approvable, it sends an 'Opinion' to the EC, the legal body that grants a community marketing authorisation. Such authorisation is valid in all Member States of the EU.

As mentioned previously, no application for marketing authorisation of a gene therapy product has yet been received by EMEA. All gene therapy products in use in the EU are so far at early stages of evaluation, that is, in phase I or phase II clinical trials. As such, they are subject to national regulations covering gene therapy in the Member States where clinical trials

are being conducted. For example, in the UK all gene therapy protocols, which include description of the product to be used and methods of its intended application, must be approved by the Gene Therapy Advisory Committee (GTAC) [21], a non-statutory government body, and the UK Medicines Control Agency (MCA) [22]. Further approvals from hospital local ethical committees and UK bodies regulating the use of recombinant DNA and the release of genetically modified organisms into the environment are also required. Similar regulatory agencies and committees operate in other EU Member States, although there is as yet little harmonisation of requirements for gene therapy products [23].

16.3 DEVELOPMENTS IN QUALITY ASSURANCE FOR BIOLOGICS: APPLICATION TO GENE THERAPY PRODUCTS

Since their introduction, biologics have brought many improvements in the prevention and treatment of disease. However, since they are prepared from biological organisms and materials, there are concerns that they may contain undesirable infectious and hazardous contaminants. These may originate in the source materials, e.g. sera, tissue extracts, or be inadvertently elicited or introduced during the manufacturing process. There have been several incidences in the past of biologics causing disease or fatalities because they contained transmissible infectious agents. In St Louis, USA, at the beginning of the twentieth century, 10 out of 11 children inoculated with diphtheria antiserum died not of diphtheria, but of a tetanus contaminant of the serum. An early polio virus vaccine prepared from monkey cell cultures was contaminated with simian virus 40 (SV40), a potentially oncogenic virus [24]. The use of pituitary gland-derived human growth hormone preparations has been associated with a number of cases of Creutzfeldt–Jakob disease (CJD) [25]. Such preparations were potential sources of the transmissible CJD agent since they derived from hundreds or thousands of unscreened pituitaries obtained from cadavers. More recently, the use of blood products sourced from unscreened donor plasmas has led to the transmission of HIV-1 and hepatitis C virus to recipients, e.g. haemophiliacs who require factor VIII [26].

These incidents clearly demonstrate that the application of appropriate tests is of the utmost importance for controlling human donor and animal sourced materials used either for isolating specific biologics or as constituents of production and/or purification processes used for the manufacture of biologics, e.g. calf serum used in the growth medium for many mammalian cell cultures, murine monoclonal antibodies used for purifying proteins. Certain measures, such as quarantining or isolation of donor animal herds,

health checks and the avoidance of the use of bovine products from countries where bovine spongiform encephalopathy (BSE) is endemic, should reduce the risk of contaminants being present in starting materials, but thorough testing by validated methods is the only way to ensure the safety of biologics.

When recombinant DNA methods were developed that led to the expression of 'therapeutic' gene products in prokaryotic cells, e.g. *E. coli*, or rodent cell lines, e.g. Chinese hamster ovary (CHO) cells, and the commercial manufacture of these biologics on a large scale, there was great concern about the quality and safety of these products. Regulatory frameworks with strict guidance for manufacturers seeking marketing authorisations for their rDNA-derived biologics were rapidly introduced by health and regulatory authorities [27,28]. The contents of these guidelines focused mainly on two key areas central to QA and safety. Firstly, the quality of all cell substrates and materials used in the manufacturing process was stressed. Particular attention was paid to eukaryotic cells, which must be shown to be free of adventitious agents, including bacteria, mycoplasma, fungi and viruses [29]. Secondly, the importance of the purification procedure was emphasised, particularly with respect to its capacity to remove as completely as possible all potentially hazardous contaminants, e.g. bacterial endotoxin (LPS), residual cellular DNA (RC-DNA) and any viral contaminants. The latter could be inadvertently propagated in the mammalian cell lines used to produce the biologic; it is known that some of these cell lines also contain endogenous viruses. That comparably severe infections to those occasionally caused by biologics prepared from naturally sourced materials, e.g. plasma, tissue extracts, have not been reported for biologics derived from large-scale fermentations of bacterial or mammalian cell cultures indicates that the measures adopted and applied to their manufacture have been successful in preventing such outbreaks. This is not, however, grounds for complacency; strict QA criteria should continue to be applied to all rDNA-derived biologics and are, in principle, applicable to gene therapy products. For example, production of replication-deficient viral vectors requires the use of special 'packaging' cell lines and these will be subject to similar QA safety considerations that apply to mammalian cell lines used in the manufacture of rDNA-derived biologics.

Special considerations are also required for the production of viral vectors. Currently, the majority of viral vectors used in gene therapy protocols are based on replication-deficient viruses [10]. The latter are propagated in special packaging cell lines, which provide viral envelope proteins to package the genome of the replication-deficient virus (Figure 16.2). This genome is defective, but contains the transgene and sequences required for transgene expression, and thus progeny virus particles, which are made by packaging genomes into viral envelopes provided by the packaging cell line, remain replication deficient for their intended target cells. Replication-deficient

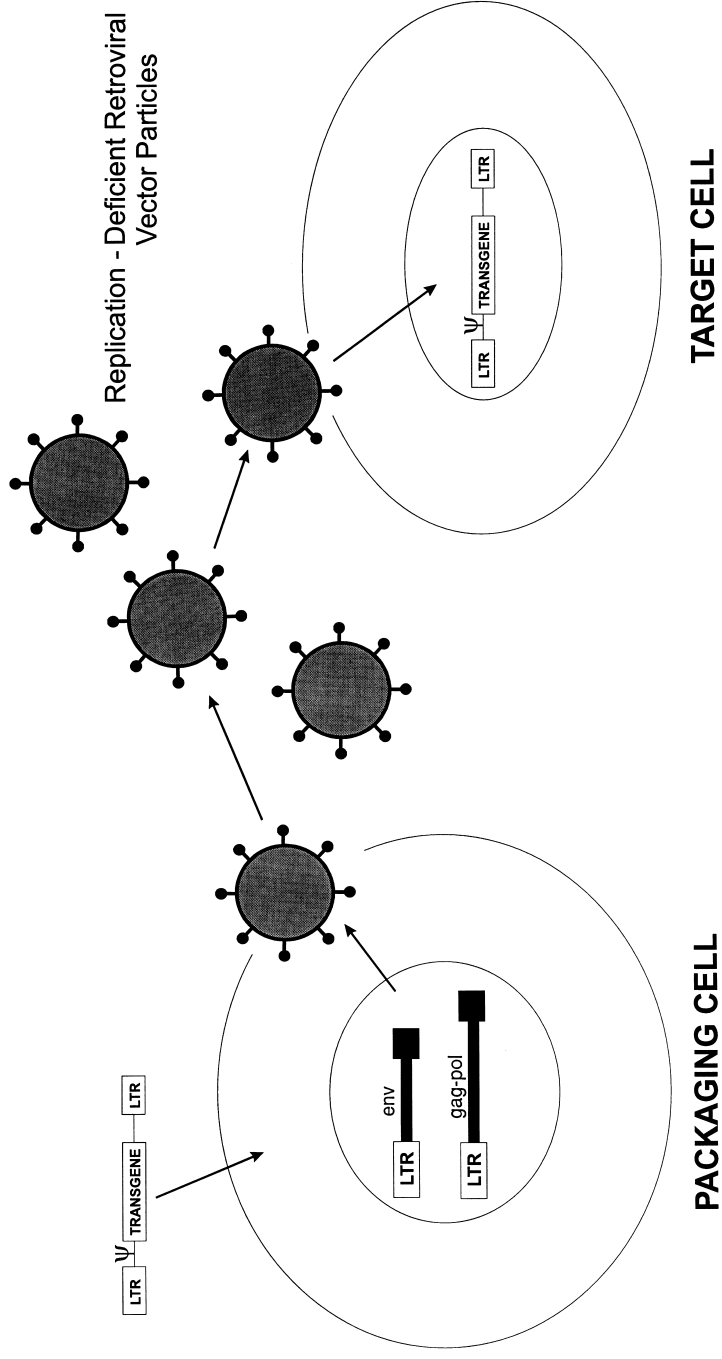


Figure 16.2. (opposite) A simplified scheme of the production of replication-deficient retroviral vectors (RDRV). In this example, a murine fibroblast cell line has been transfected with a 'helper retrovirus' genome lacking the packaging signal *psi* which is necessary to pack the retroviral RNA into viral envelopes, but containing all the other viral genes that are necessary for retrovirus replication, namely the *gag*, *pol* and *env* genes expressed from two long terminal repeats (LTRs) as shown. This transfected packaging cell has been cloned and grown as a cell line. It produces only empty retroviral capsids/particles. These packaging cells are then transfected by a second retroviral genome, this time containing the *psi* packaging signal, but lacking *gag*, *pol* and *env* genes, which are completely replaced by a transgene (therapeutic biologic-encoding gene) and its regulatory sequences. This genome is intended to be incorporated into viral envelopes encoded by the helper sequences located within the packaging cell genome. Thus, chimeric, replication-deficient, retroviral particles are produced which nevertheless can infect target cells and transfer viral genomic RNA containing the transgene and any necessary promoter sequences into them. By reverse transcription, the transgene RNA is converted into DNA and can then be integrated with target cell nuclear DNA. No new progeny retroviral particles can be made since the target cells will normally lack any viral complementary/helper sequences, i.e. that specify Gag and Env proteins.

retroviral vectors (RDRV) and replication-deficient adenoviral vectors (RDAV) can be produced by this method. The major concern is that replication-competent viruses (RCV) are not produced by recombination events with complementary viral sequences either in the packaging cells or in the target cells. The design of the defective viral genome and of packaging cells with respect to the placement of complementary viral sequences is thus paramount if the risk of producing RCV to be minimised. Additionally, there is a small risk that replication-deficient viral vectors could be rescued either by co-infection with wild-type RCV or by recombination with endogenous viral nucleic acid sequences. This could lead to vector nucleic acid mobilisation and as a consequence, non-target cells, possibly even germline cells, receiving the transgene, and also a risk of its horizontal spread to clinical staff and members of the public.

The packaging cell line may contain endogenous retroviruses or retrovirus-like particles, but, in order to maintain the desired transducing potency of the RDRV, it is usually not possible to eliminate or inactivate the unwanted viruses. In this case, safety testing must provide evidence that endogenous viruses are not harmful for the patient, i.e. that they are strictly xenotropic or that they are replication deficient.

The manufacture and use of genetically modified somatic cells also will require special considerations (Table 16.2). Genetic modification of somatic cells is readily achievable by the application of vectors to cells *ex vivo* [9,10]. In these situations, the vectors are the means or tools of gene transfer and will not normally form the final genetic medicines; these will be constituted by

Table 16.2 Quality and quality issues for cellular vehicles

Cell quality depends upon:

- Selection of donors for allogeneic cells
- Selection and control of animals for xenogeneic cells
- Characterisation of phenotype and biological activity/functionality before and after gene modification, during culture
- Quality of transducing plasmid or vector
- Stability of phenotype and biological activity/functionality in culture and after cryopreservation
- Absence of any adventitious contamination at each step of process, before and after gene modification
- High quality of reagents and biologicals used

Quality issues:

- High degree of *in vitro* cell manipulation before re-infusion (isolation, selection, expansion, gene transfer, re-selection of transduced cells, irradiation) that can take from a few days to some weeks
 - Use of plasmids or viral vectors to genetically modify the target cells
 - Use of biologics such as serum, cytokines, growth factors, antibodies and media additives for various steps in culture and selection procedures
 - Use of specialised equipment (e.g. cell sorter)
 - Cell banking or cryopreservation for future availability
-

the genetically modified somatic cells. The process of *ex vivo* manipulation and selection of somatic cells before infusion requires separate quality and safety considerations from those applying to the manufacture of vectors. The inoculation of genetically modified cells into patients is a form of somatic cell therapy, not radically different from bone marrow transplants or infusions of unmodified somatic cells. For this reason, somatic cell therapy and gene therapy have been linked together in the USA Guidelines [30]. Although this has not formally happened elsewhere, it is clear that the requirements for the manipulation of transduced somatic cells will follow the principles for handling non-transduced cells, e.g. bone marrow cells, cord blood, lymphoid cells etc., *ex vivo*, albeit in a more stringent manner. The inoculation of genetically modified somatic cells (and the implantation of neo-organs) will be governed by the same considerations of histocompatibility and immunology which apply to conventional cell and tissue transplants.

In some cases, the gene therapy product, as defined above, would be manufactured in small quantities and may be for individual applications only, thus providing a substantial difference to large-scale manufacture of current biologic products. Therefore, the general GMP guidelines [31] might also need to be adapted to this situation. A recent review issued by WHO [32] has already addressed these changes, stating that regulatory concerns are raised with respect to the expected volume and variety of 'tailor-made' products which will be individually manufactured and administered. These

will also create new needs for standardisation and control. According to the WHO review, major investments will have to be made by the authorities in bioinformatics dealing with these products and therapies.

16.4 QUALITY ASSURANCE ISSUES

16.4.1 STABILITY OF THE EXPRESSION VECTOR

If the biologic-producing cells have been genetically modified, e.g. in the case of rDNA-derived product manufacture, some quality concerns are raised in relation to the genetic stability (integrity) and transcriptional copying of the genetic material introduced into cells [27,28]. Mutated, defective or missing copies of the transgene or alterations in the regulatory sequences in some cells can lead to undesirable molecular variants of the product or can alter the yield and impurity profile. Furthermore, if the transgene is not faithfully replicated, mutations and rearrangements can accumulate with increasing cell generations, leading to increasing levels of defective product. As a consequence, toxicity, immunogenicity and potency of the product may be changed.

In order to exclude such events occurring, both the structure and the number of copies of the expression vector must be shown to have been maintained in the producer cells. The appropriate tests are carried out during the development of the transducing vector and the producer cells, in the validation phase of development and, with a more limited range of tests, on a regular basis during production. During developmental stages considerable attention should be paid to the characterisation of the structural integrity of the transducing vector and, in particular, the expression construct contained within it. It is important that the quality-determining characteristics established during development remain unchanged in the producer cells. Limits should be applied to the population doublings of cells, especially if there is evidence indicating that the quality of the product is compromised by prolongation of the manufacturing process.

Issues relating to the genetic stability and faithful copying of genetic material(s) also apply to gene therapy products [17,30]. For example, the capacity of an amplification process to faithfully generate multiple copies of the genetic material (transgene, regulatory sequences and other vector sequences) must be assessed. In addition, the extent to which recombination and mutation can occur within the transgene and its regulatory sequences during replicative procedures should be monitored. However, in some cases, copy errors, mutations and recombinations will be difficult to control, e.g. in the production of RDRV, and in such circumstances product safety and the risk:benefit ratio of the intended treatment must be seriously considered.

16.4.2 PRODUCER CELL- AND VECTOR-DNA

When rDNA-derived biotechnology medicinal products were first developed, concerns were raised over the amounts of residual nucleic acids, particularly DNA deriving from producer cells, that remained in the final product [27,28]. This was mainly because such DNA, especially that from human or mammalian cells, could contain potentially oncogenic sequences and give rise to the risk of tumour development in recipients of these products. The issue of RC-DNA has been addressed at length by regulatory authorities. Relevant experimental findings were considered by a WHO Study Group [33] leading to the establishment of extremely low limits for RC-DNA amounts and thus to a relatively huge safety margin. However, in the light of experience in the manufacture and application of rDNA-derived products, the risks associated with the presence of RC-DNA impurities are being reconsidered: many experts now believe that these risks are less serious than originally assumed [34,35].

Gene therapy products that are derived from cells are also subject to this issue. For example, cells which are transduced *ex vivo* by RDRV will be exposed to variable amounts of packaging cell DNA since the RDRV preparation is not in many cases purified from packaging cell supernatant, so as to preserve infectivity. Uptake of RC-DNA by target cells may pose an additional risk of cellular transformation on top of that engendered by random integration of vector nucleic acid into the genome of target cells [17,30]. Where possible, changes in cellular morphology, adherence characteristics and proliferative responses of transduced cells should be monitored following vector transduction and other *ex vivo* manipulation procedures. Any uncertainties with regard to cellular transformation to the neoplastic phenotype and the possible infusion of potentially tumorigenic cells into patients must be considered in relation to the seriousness of the disease treated.

16.4.3 THE MANUFACTURING PROCESS

From the very beginning, the manufacture of biologics has focused on following a consistent process ('the process is the product' concept). Although, via the GMP regulations [31], this principle has been introduced as a general requirement, QA in the field of biologics is still more process orientated than in the manufacture of chemical drugs [36].

The manufacture of most biologics may be divided into the fermentation and the purification parts. The latter is relevant for practically all products derived from prokaryotes or eukaryotes, especially continuous cell cultures. In general, both process parts are relevant to the manufacture of gene therapy products.

16.4.3.1 Cell Banking System

If biologics are produced by biotechnological processes involving cells (prokaryotes or eukaryotes), it is a general principle to establish an appropriate cell banking system consisting of a well-characterised master cell bank (MCB) and working cell banks (WCB). These cell banks must be fully characterised with regard to distinguishing genotypic, phenotypic and biological markers, which are useful for establishing the identity of the cells. They are also fully tested for the integrity of plasmids and/or stability of plasmid/expression construct numbers and/or product yield and the absence of adventitious microorganisms, such as viruses and mycoplasma. All these measures contribute to consistent provision of uniform and safe producer cell cultures for starting production runs. Such criteria also apply to the cells required for the manufacture of gene therapy products, including cells for DNA, plasmid or vector production. Cell banking of transduced, genetically modified cells may be necessary where such cells are grown to large numbers and storage is required prior to infusion into patients, or where cells are required from time to time to prepare neo-organs or encapsulated cell cultures.

16.4.3.2 Fermentation

The fermentation process itself is a potential source of contaminants, including RC-DNA, RC-protein and unwanted components of the fermentation medium. The constituents of the fermentation medium must be carefully selected with respect to purity and safety. For example, it is undesirable to use substances such as penicillins, which are known to provoke adverse reactions in certain individuals. Avoidance of bovine serum sources from countries reporting BSE in cattle herds is an important new requirement. Where potentially hazardous components cannot be avoided, the purification process must have the capacity to remove them, or at least reduce them to the lowest levels, where a large safety margin can be assured.

It is essential that all fermentation steps are clearly defined and fully validated according to GMP conditions [31] and that in-process controls are established and strictly followed. These should remove or reduce to a minimum process variability which could lead to unintended changes to the product itself, or cause undesirable qualitative and quantitative fluctuations in the impurities/contaminants present in the fermentation medium. Unwanted variations in the fermentation may result in undesirable post-translational modifications to the product, including N- and O-glycosylation, acetylation, hydroxylation, gamma carboxylation, deamidation and oxidation of SH groups. It is well known that carbohydrate composition and

structure, for example, play an important role in determining the activity of glycoproteins, particularly *in vivo*.

The fermentation process should take place within set physical and temporal limits based on knowledge of scale-up procedures, viability of cells under large-scale culture conditions, stability of product expression, etc. Criteria, e.g. low yields of product, for the rejection of harvests should be defined.

In general, proof of consistency of the fermentation process requires full analysis of three or more production runs. Consistency has to be demonstrated by validation studies demonstrating the uniformity of pivotal process parameters, such as maintenance of yield, consistency of cell growth, production rate, pH, oxygen content and producer cell characteristics at the end of production and beyond.

The principles of GMP and in-process controls that apply to fermentation processes to manufacture rDNA-derived therapeutic medicinal proteins also apply to the fermentation processes involved in the large-scale preparation of gene therapy products, especially viral vectors. These processes must be highly reproducible so that a consistent product is made, especially when the product, e.g. RDRV-containing preparations, cannot be subjected to extensive purification procedures.

16.4.3.3 Purification

The methods used for purification should be described in detail and must be validated. The combined stages of product purification must be capable of removing impurities/contaminants, including RC-DNA and RC-proteins, undesirable components of the fermentation medium, e.g. bovine serum albumin, methotrexate, insulin, carbohydrates, lipids, product-related proteins (dimers, aggregates, degradation products) and any endogenous virus of the producer cells, as well as any adventitious agents originating from the components of the medium or materials used during purification, e.g. monoclonal antibodies, to levels below the limits adjudged to be safe. Attention must also be paid to ensure that materials used for purification, e.g. materials used for affinity chromatography, do not compromise the quality of the product.

The need for validation studies has already been emphasised. It relates to both the fermentation and the purification stages. In the case of purification validation studies are required to determine the capacity of purification steps for removing the expected and potential impurities and contaminants. They include the use of a carefully selected group of viruses which exhibit a range of physicochemical features relevant to their behaviour on purification [37].

In principle, the considerations described above will also apply to gene therapy products. If, for example, plasmid DNA is the product then the

purification process must be effective enough to remove contaminants, particularly toxic and antigenic substances. If, however, replication-deficient viral vectors are produced, only limited purification may be possible and in some cases, e.g. retroviral vectors, the product essentially consists of a crude cell supernatant containing viral vector particles. In the latter case, safety testing should reflect the intended use of the product. Measures should of course be taken to ensure such products are consistent, for example by applying rigorous control of fermentation processes involved in vector production.

16.4.4 SPECIFICATIONS AND QUALITY CONTROL

The first biological medicines used to treat patients were usually complex mixtures of biological materials containing only small quantities of active substances. Frequently, no details were known about the chemical structure of the active principle. These products could only be specified in terms of safety and minimum potency [38]. However, the passage of time has brought the development of both effective purification procedures and powerful analytical methods, and thus the means of characterising biologically active substances to a high degree. Parallel to this progress in technology came regulations requiring the tightening of product specifications, in particular purity requirements. For instance, stringent requirements for rDNA-derived products were quickly established as soon as they became available for clinical use.

Today, analytical technology has been extensively developed, and a wide range of methods covering identity, purity, potency, structure, etc., is generally applied during product development and in routine quality control. The analytical methods applied nowadays range from basic spectroscopic, electrophoretic, immunochemical and chromatographic methods to highly sophisticated techniques. These include circular dichroism (CD), surface plasmon resonance (SPR), nuclear magnetic resonance (NMR), mass spectrometry, gas chromatography-mass spectrometry (GC-MS), and Fourier transform infrared (FT-IR) Raman spectroscopy.

The quality requirements for a product should reflect its intended clinical use. Thus a preparation that is to be administered over a long period in large doses is likely to need careful testing for traces of antigenic contaminants. Different criteria might justifiably apply to a product that is used only once in a life-threatening condition. Guidance for setting specifications is given by the appropriate guidelines [39,40].

16.4.4.1 Safety and Purity

Contaminants of biotechnology medicines mainly originate from the producer cells and the materials used in the manufacturing process, and testing

should therefore reflect the nature of expected contaminants. These include substances of producer cell origin such as RC-DNA and RC-proteins, molecular variants of product due to genetic instability or unintended variations in the manufacturing process, materials that have been added during fermentation or purification, and adventitious infectious agents, including viruses, mycoplasma or any other microorganism. Tests to detect these contaminants should be highly sensitive, with defined limits of detection. Tests to show freedom from contaminating adventitious agents are essential prior to cell banking. Further testing during the manufacturing process and at various purification stages, including the final bulk product, is important. Finally, the finished product should comply with the requirements of EC Directives and pharmacopoeias; where this is not possible the omission of tests should be justified by the manufacturer [27].

In the field of gene therapy products, specifications for safety and purity must be defined, taking into consideration their intended use. For example, plasmid DNA itself should not induce an immune response and, therefore, should not contain antigenic contaminants or substances acting as adjuvants. However, no stringent purity requirements can be defined for retroviral vector suspensions in terms of producer cell derived protein and DNA, but adequate safety testing is essential.

16.4.4.2 Biological Activity (Potency)

The ICH Guideline on specifications [39] defines potency as the 'specific ability or capacity of a product to achieve its intended biological effect'. It is the quantitative measure of biological activity and should be based on the attribute of the product which is linked to relevant biological properties. Biological activity is measured in bioassays. Where possible, the biological activity measured should be relevant for the intended clinical use of the product, but, although preferable, is not expected to mimic the clinical situation.

It is sometimes suggested that bioassays are performed for historical reasons only, initially justified by a lack of knowledge about both the exact nature of the active principle and appropriate biochemical testing methods. However, in spite of increasing knowledge and progress in technology, bioassays are still important tools in the quality control of biologics. It is characteristic for many biologics that molecular variants and other product-related molecules may arise, such as glycosylation isoforms, oxidised or deamidated molecules, and aggregated product molecules. Biochemical methods may be capable of detecting and quantifying such undesirable impurities but, apart from clinical studies, their significance for the product's potency can only be assessed by means of bioassays. This is only one reason for the fact that, in spite of technological progress, bioassays are still indis-

pensible for the quality control of biologics. Furthermore, they are important for monitoring product consistency and stability [29].

According to the ICH Guideline [39], bioassays may be classified as follows:

- Animal-based assays, in which a biological response to the product is measured.
- Cell line based assays, which measure a physical, biochemical or physiological response.
- Biochemical assays, which measure a biological activity such as enzymatic reactions or direct biological responses induced by immunochemical interactions.
- Other methods, such as ligand/receptor binding assays.

Particular attention is being paid to animal-based bioassays, where alternative cell-based assays should be sought in the light of cost-effectiveness and animal protection legislation. The relevant guidelines [39,40] state that the requirements for an *in vivo* bioassay may be relaxed 'when sufficient correlation studies between physicochemical or *in vitro* bioassays have been carried out showing that estimates based on *in vitro* tests are sufficiently precise and accurate'.

Emerging products in the field of gene therapy, such as viral vectors or complexed nucleic acids, will also need potency testing as proof of their efficacy. With regard to the analytical methods available today and taking account of the complexity of those products, functional assays, such as the transfection of cell lines, have to be performed and quantified. Guidance for and standardisation of testing methods and statistical evaluation are required.

16.4.4.3 Regulatory Aspects, Standardisation and Batch Release

The historical reasons why biological medicines have been subjected to surveillance and batch release by national health authorities have already been addressed. Institutions which were qualified to execute the specific responsibilities were founded in most countries at the beginning of the twentieth century, frequently in response to the incidents described in Section 16.3. Examples of such institutions are the Paul Ehrlich Institute in Germany, the National Institute for Biological Standards and Control (NIBSC) in the UK and the Division of Biologics Standards of the NIH in the USA, which was the predecessor of the Center for Biologics Evaluation and Research (CBER).

These control authorities were established for testing and evaluating biological medicinal products and for controlling their distribution by batch

release, in order to guarantee their safety and efficacy. As a consequence of analytical and technological progress, certain authorities, e.g. the US FDA, have undertaken to distinguish between conventional biological products and 'well-characterised products', mainly derived from rDNA and hybridoma technologies. The latter benefit from reforms in legislation and are no longer subjected to batch release [41,42]. A number of EC Directives have also moved in this direction and the likelihood is that in future blood products and vaccines will still need batch release whereas other biotechnology medicines could be adequately controlled through a system of lesser checks [32].

In addition to these tasks, some control authorities also play an important role in the field of international biological standardisation. One of the largest problems to cope with in the testing of biologics is the reproducible measurement of potency and its reporting in a universal unitage. To address these problems, reference preparations for biologics have been established by WHO and play an important role in the calibration of bioassays and reliable comparison of the activities of biologics from different manufacturers worldwide. Therefore, WHO's Biological Unit and its Expert Committee on Biological Standardization (ECBS) have undertaken to establish International Standards (IS), which are developed, held and distributed by WHO-affiliated International Laboratories for Biological Standards, principally the NIBSC in the UK [32]. Standardisation of certain gene therapy products will almost certainly become necessary. The NIBSC, with its wide expertise in the testing of medicinal biological products, will be in a position to play a major role in providing advice and in meeting the new challenges involved in the standardisation and testing of gene therapy products.

16.5 SAFETY ISSUES

The quality assurance of gene therapy products has already been addressed by specific guidelines [17,30]. According to these, the general requirements valid for biologics will also apply to the new products of gene therapy, but new safety issues will have to be dealt with. These include:

- Genetic recombination allowing viral vectors that have been 'engineered' to be replication deficient to become replication competent.
- Mutations in viral vector genomes, particularly in the contained transgene.
- Unpredictable effects by random integration of vector nucleic acid into chromosomal DNA (e.g. by interfering with the expression of a tumour suppressing gene).
- Possible changes in cell behaviour (e.g. heightened immunogenicity).
- The possibility of the transgene getting into the germline.

Some of these, e.g. genetic recombination, mutations and changes in cell behaviour, should be minimised if appropriate QA measures are taken during the manufacturing process. Nevertheless, as with biologics, thorough safety testing at the preclinical stage is mandatory to give assurance that gene therapy products are safe. The CPMP has considered that the issues and principles applying to biotechnology medicines are relevant to gene therapy product safety [43,44]. There are many different approaches to preclinical safety testing (see [43,44] for further information), but a strong scientific rationale should be developed that is product related. Safety assessment using preclinical animal models must address the suitability and relevance of the animal species and physiological/disease state in relation to how the product is intended to be used in patients. Ideally, the product should elicit the same or similar biological responses to those expected in humans. If testing for RCV, the animals selected should show similar sensitivity to infection by such viruses as shown in humans. Regarding the use of genetically modified allogeneic or xenogeneic cells, animals studies may provide useful information about any 'toxic' complications arising out of unwanted immune responses. Routine animal toxicology studies (single-dose toxicity, repeated-dose toxicity, immunotoxicity, reproductive toxicity etc.) may give information concerning the potential to cause undesirable or unexpected effects, but studies should be designed on a case-by-case basis. It may be necessary to consider the use of transgenic animals or immunodeficient animals bearing a human tissue transplant to overcome problems of safety assessment due to lack of activity in normal animals. Where possible, the long-term effects of continuous expression of the gene product encoded in the transgene should be addressed in suitable animal studies. There may be unexpected/undesirable consequences arising from long-term transgene expression, e.g. the triggering of autoimmunity due to overstimulation of immune responses. As yet, there is not a great deal of experience in this area, but further experimental and clinical studies should provide clearer indications of the safety concerns that need to be addressed when transgene expression is maintained over long periods of time.

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17 Development and Regulation of Gene Therapy Drugs in Germany

KLAUS CICHUTEK

17.1 INTRODUCTORY REMARKS

In Germany, gene therapy drugs are regulated in a similar way to other medicinal products [1–5]. Regulation is thus mainly provided by the German Drug Law (*Arzneimittelgesetz*, AMG [6]) and, in certain cases, by the professional law of physicians (*Ärztliches Standesrecht*). In contrast to conventional drugs, gene therapy products are not only produced using recombinant DNA technology, but may also be made of or contain genetically modified organisms (GMOs). Therefore, regulations of the German Gene Technology Law (*Gentechnikgesetz*, GenTG [7]) are applied to the preclinical research and development stages of gene therapy. However, the application of GMOs (which can be ingredients of gene therapy drugs) to human beings is not regulated by the GenTG. For the clinical use of gene therapy drugs, approval of deliberate release according to the GenTG is not required. Regulations to assure protection from infection of other human beings apart from the patients themselves and environmental risks are provided by the German Law on Protection from Epidemics (*Bundesseuchengesetz*). In summary, clinical studies using gene therapy drugs can be initiated in Germany within a general time frame of less than 6 months.

Marketing authorisation is provided by the European Commission (EC) in a central European process [8] coordinated by the European Medicines Evaluation Agency (EMA). It includes review of data, allowing the assessment of the quality, safety, efficacy and environmental risks of the drug as obtained during clinical trials.

The medical treatment of humans with drugs in Germany does not require any form of approval from competent authorities in Germany comparable with approvals of Investigational New Drug Applications (INDs) or phys-

ician-sponsored INDs as provided by the Food and Drug Administration (FDA) in the USA [3–5,9]. Instead, a positive appraisal of the local ethics committee in conjunction with the appraisal of the central Committee for Somatic Gene Therapy [10] and notification, as well as supervision by competent authorities of the relevant *Land*, are in general obligatory. There are several laws, directives and guidelines applicable to different aspects of a clinical gene therapy trial. This ensures that international and national standards developed for the manufacture and clinical use of gene therapy drugs are observed (Table 17.1). In many cases, different competent authorities of the relevant *Land* have to be contacted [6,7], but generally these legal processes preceding the initiation of the manufacture of a drug or a clinical trial are relatively informal and can be completed in a reasonable time period. Advice can be obtained from these regional authorities as well as from the federal higher authorities. In addition, scientific advice on the development and clinical use of gene therapy drugs is offered by the central Committee for Somatic Gene Therapy of the German Medical Association.

17.2 DEFINITION

As yet, the term ‘gene therapy product’ has not been officially defined in the AMG. However, a reasonable definition has been published in the final report of the German working group on Somatic Gene Therapy (*Bund/Länder-Arbeitsgemeinschaft ‘Somatische Genthérapie’*) in 1998 [1], which described the state-of-the art of gene therapy and resulting ethical and regulatory considerations for Germany. In this report, gene therapy drugs were defined as follows: ‘Somatic gene therapy drugs for human use either consist of or contain genetically modified cells or are intended to be used for the genetic modification *in vivo* of human somatic cells’. According to this definition, genetically modified autologous, allogeneic or xenogeneic cells, as well as viral or non-viral vectors, are gene therapy products (Figure 17.1). More importantly, it is clarified by this definition that only those gene therapy products which are intended for *in vivo* use are drugs. Consequently and as stated above, the regulations of the AMG are applied. Moreover, drugs can be used for therapy, prophylactics and *in vivo* diagnosis (§2 AMG).

Prior to marketing authorisation, medicinal products, here often referred to as drugs, are generally used in patients during clinical trials (phases I to III). Finished drugs will require marketing authorisation according to Council Regulation (EEC) No. 2309/93 [8] in a centralised European procedure, whereas marketing authorisation is not required for individually prepared drugs. Vectors or DNA used *ex vivo* for the preparation of gene therapy drugs are only active ingredients or components of the drug. Thus, marketing authorisation of these components or ingredients is not required.

Table 17.1 Regulations applying to the manufacture and clinical use of gene therapy (GT) drugs in Germany prior to marketing authorisation

International standards	European regulations, directives and other guidelines	Specific regulations applied in Germany
GCP	Council Directive in preparation, ICH guideline	Drug Law (AMG)
GMP	Council Directive 91/536/EEC, European Note for Guidance, WHO guideline, PIC guideline	Operation Ordinance for Pharmaceutical Entrepreneurs
GLP	Council Directives 87/18/EEC and 88/320/EEC, guidelines (OECD)	Law on the Use of Chemical Substances (Annex 1 of §19 ChemG)
Contained use	Council Directive 90/219/EEC	Gene Technology Law (GenTG)
Environmental risk assessment	Council Directive 90/220/EEC, Council Regulation No. (EEC) 2309/93	Law on Protection from Epidemics (<i>BundesseuchG</i>)
Manufacture of GT products	European Note for Guidance 'GT products . . .' and 'Safety Annex', others	'Guidelines for the gene transfer into human somatic cells', German Medical Association
Pharmacological/toxicological tests	Council Directive 75/319/EEC, European Note for Guidance 'GT products . . .' and 'Safety Annex', others	Official Drug Testing Guidelines (<i>Arzneimittelprüfrichtlinien</i>), 'Guidelines for gene transfer into human somatic cells', German Medical Association

The definition of gene therapy drugs as given above formally includes genetically modified xenogeneic organs intended to be used as human transplants. However, it seems likely that more specific regulations may be found during the next years for these xenotransplants. These regulations may, however, follow the general concept that has been applied for the regulation of gene therapy in Germany (see below). Genetically modified xenotransplants are not regulated by the German Transplantation Law (*Transplantationsgesetz*).

Viral vectors used in gene therapy are most often replication incompetent because they are used as gene transfer vehicles. Replication-competent vectors such as the ONYX vector (an adenovirus variant supposed to exclusively replicate in and kill tumour cells) would also be gene therapy products, if their use is intended to result in the genetic modification of somatic cells for a specific therapeutic purpose, here to eliminate malignant tumour cells. Consequently, the vectors are readily distinguished, for example, from live

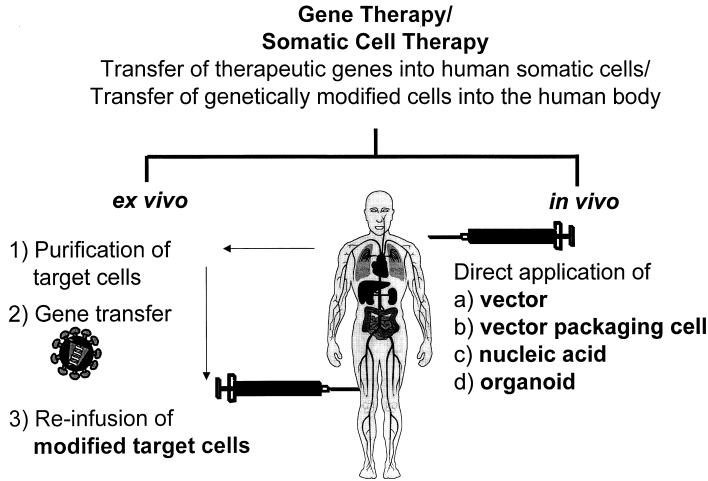


Figure 17.1. *Ex vivo* and *in vivo* treatment of patients during somatic cell and gene therapy. The gene therapy drugs used for treatment, prophylaxis and *in vivo* diagnosis in human patients are shown in bold.

attenuated viruses used for prophylactic vaccination [2,11] because the latter are used to induce an immune response against the virus itself. In gene therapy, it is most often intended to avoid immune responses against the vector particles.

17.3 PRECLINICAL RESEARCH AND DEVELOPMENT

Research and development in gene therapy necessarily includes recombinant DNA technology. As GMOs are constructed or used, research has to be performed in facilities that have been registered as gene laboratories at the competent authority of the *Land* (GenTG), which is often a so-called *Regierungspräsidium* (Figure 17.2). Initial registration of the facility in Germany always has to include the registration of the recombinant experiments or genetic operations to be performed (*gentechnische Arbeiten*). The safety level of the gene laboratory and the safety level of the operations to be performed are defined by the risk group of the GMOs to be used. For example, retroviral or adenoviral vectors used in gene therapy generally fall under safety level 2. First-generation lentiviral vectors, if derived from human immunodeficiency virus (HIV), currently fall under safety level 3, whereas non-viral vectors which do not contain any GMOs are handled under safety level 1 if used together with other safety level 1 GMOs. Once the gene transfer has occurred, safety level 1 is sufficient to handle, store or inactivate genetically modified

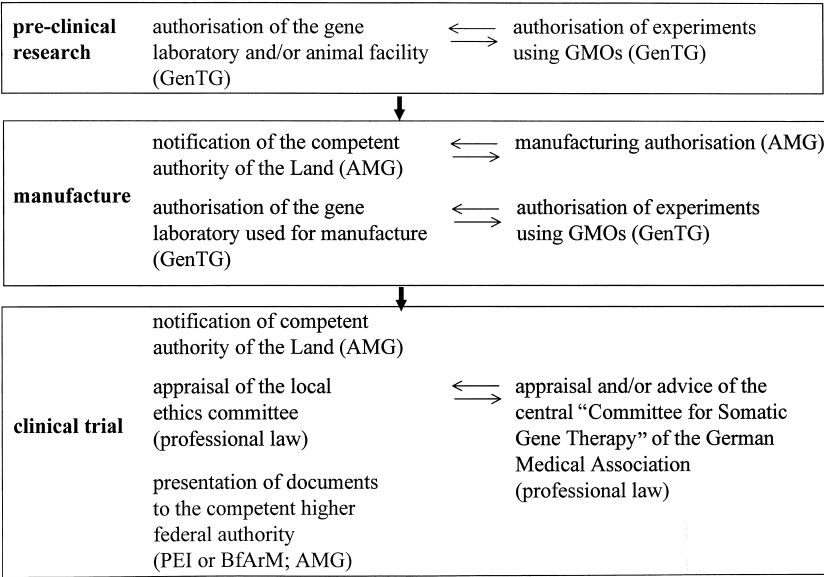


Figure 17.2. Steps to be taken in order to preclinically test, manufacture or clinically use gene therapy drugs in Germany.

human cells, if taken from patients who had been shown to be seronegative for pathogens like HIV or hepatitis B virus (HBV).

Forms needed to notify the competent authority or to register the gene laboratory and the genetic operations are available from the competent authority (GenTG), which also gives advice before or during the registration procedures. For the construction or use of gene laboratories falling under safety level 1, only notification of the competent authority of the *Land* where the laboratory is located is necessary. Notification can be followed by immediate initiation of the experimental work. For additional genetic operations, i.e. new, coherent series of recombinant experiments leading to the construction or use of one or more comparable GMOs, only precise records have to be kept and no additional notification of the authority is necessary. In contrast, registration of gene laboratories of safety levels 2 and 3 require an application and authorisation by the competent authority of the *Land*, which can take up to 1 and 3 months, respectively. In addition, each new and additional genetic operation in a facility that had already been registered requires authorisation within similar time periods. The recommendations [12] by the German Central Commission for Biological Safety (*Zentrale Kommission für die Biologische Sicherheit*, ZKBS), the secretariat of which is located at the Robert-Koch-Institut in Berlin (Internet address: <http://www.rki.de/GENTEC/GENTEC.HTM>), on the assignment of risk groups to specific

GMOs may be taken into account. This Commission, however, is only an advisory commission and the authority of the *Land* has the final right of decision. Therefore, legal guidance is normally given by the competent authority of the *Land*.

17.4 MANUFACTURE OF DRUGS AND INGREDIENTS FOR CLINICAL USE

According to §67 AMG, notification of the competent authority (AMG) is required for companies or establishments which develop or manufacture drugs, subject drugs to clinical trial or residue tests, test, store, package or market them or are otherwise engaged in trade with drugs. The competent authority of the *Land* will give advice on the information to be included in the notification.

Alternatively, the manufacture of the gene therapy drug or the active ingredient may have to be authorised (§13 AMG). In those cases where the person manufacturing the drug and the physician treating one or more patients with it are not one and the same person, this authorisation is required. Authorisation is also provided by the competent authority of the *Land* (AMG). For a clear distinction of the legal requirements, the competent authority (AMG) of the *Land* where the clinical trial is to be performed can be consulted.

Only when a company or establishment is manufacturing drugs or test preparations of a drug intended to be used for the treatment of a larger number of patients and distributes this drug professionally to physicians not involved in the development or manufacturing, is a control of the manufacturing process and establishment by a governmental authority supposed to assure the application of all necessary standards. In all cases, however, the physician using the drug for the treatment of patients is still fully responsible for it, i.e. he or she has to be fully informed about its quality and safety. In addition, the physician has also to obtain information about the pharmacological/toxicological data obtained in preclinical experiments or other clinical trials already performed. In order to make sure that manufacturing of a defined drug is authorised, the process descriptions have to be included in the application for manufacturing authorisation (§14 (1) Nr. 6 AMG) and will be examined by the competent authority. This authority is also in charge of supervising the manufacture and has the legal power to stop it, if irregularities are found (§64 AMG).

Manufacturing authorisation for those gene therapy products which are sera, vaccines or blood products is given by the competent authority (AMG) of the *Land* under consultation with the Paul-Ehrlich-Institut (PEI; §13 AMG), which is the competent higher federal authority (§77 AMG). In practical

terms the documents supporting manufacturing authorisation are examined by the PEI. In addition, a regulatory scientist from the PEI will together with the competent authority of the *Land*, examine the manufacturing facilities. This procedure should ensure manufacturing according to the standards in the German directive Operation Ordinance for Pharmaceutical Entrepreneurs [13]. This ensures that similar standards as those defined by GMP are applied to the manufacture of a product for clinical use. As with all biological drugs, not only the final quality of the product, but also full control of the manufacturing process (in-process controls) have to be assured and high quality ingredients have to be used. The PEI is consulted by the competent authority of the *Land* for advice on special biological products such as gene therapy drugs in order to recruit expert knowledge and experience. Similarly, the Federal Institute for Drugs and Medical Devices (BfArM) can also be consulted by the competent authority of the *Land*.

If the manufacture of a gene therapy drug or its recombinant components includes the generation, inactivation, storage or use of one or more GMOs, the facilities and the operations have to be registered at the competent authority of the *Land* (GenTG), as explained above.

17.5 CLINICAL TRIALS OF GENE THERAPY DRUGS

According to the current 'Guidelines for gene transfer into human somatic cells', published by the German Medical Association, gene therapy drugs in their current state of development should be used only during clinical trials rather than during compassionate use regimens (Figure 17.2). Before initiating a trial, the competent authority of the *Land* (AMG) has to be notified (§67 AMG). This authority also supervises the trial (§64 AMG) and would be able to stop it, if irregularities were to occur. In addition, the (positive) appraisal of the local ethics committee, which has to be formed according to the laws of the *Land* where it is located, has to be obtained (§40 (1) Nr. 6 AMG). According to the 'Guidelines for gene transfer into human somatic cells' published by the German Medical Association, the central Committee for Somatic Gene Therapy should also give an appraisal (Commission for Somatic Gene Therapy (KSG-BÄK), Wissenschaftlicher Beirat der Bundesärztekammer, Herbert-Lewin-Str. 1, D-50931 Koeln, Tel. + 49 221 4004 0, Fax + 49 221 4404 386, email dezernat6@baek.dgn.de). Normally, this committee is contacted by the local ethics committee. However, the sponsor or the principal clinical investigator may also contact the central committee directly and ask for an appraisal. The applicant and the local ethics committee will then be informed in parallel about the decision of the committee. The application sent to the central ethics committee should be based on the guidelines of the German Medical Association and should contain answers to the questions given in

appendix A of the guidelines [10]. The central committee also gives advice on scientific or ethical problems of planned clinical gene therapy trials. For this, the applicants can be invited to the quarterly meetings of the committee.

Finally, documents defined in §40 AMG are presented in written form to the competent higher federal authority (AMG). The federal higher authority for those gene therapy drugs which are vaccines or blood products, which includes drugs for blood stem cell transfer and cancer immuno-therapeutics, is the PEI in Langen (Prof Dr K. Cichutek, Department of Medical Biotechnology, Paul-Ehrlich-Institut, Paul-Ehrlich-Str. 51–59, D-63225 Langen, Tel. +49 6103 77 5307, Fax +49 6103 77 1255, email cickl@pei.de). For other gene therapy drugs, the federal higher authority is BfArM in Berlin (Dr U. Kleeberg, BfArM, Seestr. 10-11, D-13353 Berlin, Tel. +49 30 4548 3356, Fax +49 30 4548 3332, email u.kleeberg@bfarm.de). The presentation simply encompasses the formal submission of defined documents. The submission is followed by an acknowledgement of the federal higher authority, which also gives a registration number to each trial. If the documents are complete, a positive appraisal of the relevant local ethics committee, the study design (*Prüfplan*) and sufficient pharmacological/toxicological data had been included (see §40 AMG for details), the trial can be initiated. Forms for the presentation are available via the Internet (<http://www.pei.de/inhalt1.htm>; follow the 'Service&Aktuelles' link, then 'Service-Links', then the Vorlageblatt 'Klinische Prüfungen' link). A general outline of the pharmacological/toxicological data which are necessary may be derived from the German Official Drug Testing Guidelines (*Arzneimittelprüfrichtlinien* [14]). More specific information is also given in the European Note for guidance 'Gene Therapy Products – Quality Aspects in the Production of Vectors and Genetically Modified Somatic Cells' and its safety annex [15] or similar Directives and Guidelines [16–20]. More detailed information is currently not available for gene therapy drugs. In general, the pharmacological/toxicological data should comply with the presently prevailing standards of scientific knowledge.

In §40 ff. AMG the conditions under which a clinical trial has to be performed are defined. Basically, all responsibility remains in the hands of the physician who is treating the patients, more specifically the principal investigator (*Leiter der klinischen Prüfung*). Risk sharing between the competent authorities, the clinician and/or sponsor of a trial is not compatible with the current legal system.

17.6 ONGOING OR PLANNED GENE THERAPY TRIALS IN GERMANY

Currently, there is no official or public registry of gene therapy trials in Germany. The information obtained by the federal higher authorities is

confidential and is not available to the general public. However, the German Working Group for Gene Therapy (*Deutsche Arbeitsgemeinschaft Genterapie*, DAG-GT; contact: Dr M. Hallek, Muenchen, email: hallek@lmb.uni-muenchen.de), an association of scientists and clinicians involved in gene therapy, is encouraging registry of the so-called gene therapy protocols at the German Gene Therapy Register (*Deutsches Genterapie-Register*, DGTR) at the German Working Group on Gene Therapy (DAG-GT). The protocols should also be published in the *Journal of Molecular Medicine*. Detailed information can be obtained from the Working Group.

General information about ongoing or planned gene therapy trials in Germany is provided in Table 17.2. This table contains the information available from the competent federal higher authorities and the German Medical Association, and thus contains an accurate description about the situation of clinical gene therapy in Germany. Since the first two gene therapy treatments were performed in Germany in 1994, clinical trials have been performed with a number of gene therapy drugs. Initially, autologous tumour vaccines prepared using retroviral vectors *ex vivo* were often used. Later, viral vectors were also applied *in vivo* to promote apoptosis of tumour cells. In addition, more and more non-autologous drugs were used, which are finished drugs and would require marketing authorisation. Most of the clinical trials, however, are still concerned with drugs for the treatment of cancer and are in phase I or II. It is also notable that a clinical trial involving the use of a gene therapy drug for the treatment of rheumatoid arthritis has been initiated. This is one of the few applications of somatic gene therapy for the treatment of a non-life-threatening disease.

17.7 INVOLVEMENT OF THE PAUL-EHRlich-INSTITUT IN GENE THERAPY

Marketing authorisation for gene therapy drugs will have to be obtained through the centralised procedure defined by Council Regulation (EEC) No. 2309/93. Data supporting marketing authorisation can be included in a single file and forwarded to the EMEA. The EMEA's Committee for Proprietary Medicinal Products (CPMP) appoints a rapporteur and a co-rapporteur from its members. At present the German CPMP members are recruited from the PEI and BfArM. The rapporteurs appointed for review of applications will prepare assessment reports based upon evaluations from selected experts. On the basis of the reports, the CPMP will come to a recommendation concerning the licensing of a drug for the European Commission. In order to achieve short review times and a successful application, companies may choose to involve potential rapporteurs or experts in early discussions about the design of clinical trials and other relevant matters. Applicants can also

Table 17.2 Ongoing or planned clinical trials using gene therapy drugs in Germany (1994 to January 1999)

Drug	Disease	Transfer vector/ method	Approach	Number trials
Autologous tumour vaccines	Malignancies of the haematopoietic system, malignant melanomas, renal and pancreatic tumours	Retroviral vector, transfection	<i>Ex vivo/ in vivo</i>	7
Non- autologous tumour vaccines	Malignancies of the haematopoietic system, malignant melanomas, renal and pancreatic tumours	Retroviral vector, transfection	<i>Ex vivo/ in vivo</i>	8
Tumour cell killing drugs	Brain tumours, lung cancer	Adenoviral vector, cells releasing retroviral vectors, encapsulated cells	<i>In vivo</i>	10
Cell marking drugs	CML, AML, multiple myelomas	Retroviral vector	<i>Ex vivo</i>	4
Others	Rheumatoid arthritis, diabetes, others	Retroviral vectors, DNA	<i>Ex vivo/ in vivo</i>	3

propose to the EMEA suitable rapporteurs for the evaluation of their applications.

The PEI was proposed by the German Working Group on Gene Therapy as the federal higher authority competent for gene therapy drugs in Germany. Thus a number of sponsors of clinical trials, investigators or manufacturers of gene therapy drugs have already been seeking advice from the PEI on the planning of clinical trials, the testing of the safety and quality of gene therapy drugs, and other problems concerning their future marketing authorisation. The current involvement of the PEI in gene therapy regulation is shown in Figure 17.3. Current research projects include the generation of retroviral cell targeting vectors for human T cells and blood stem cells as well as the development of a primate gene therapy model for the treatment of AIDS.

There is also a process coordinated by the EMEA which may help to reduce the time needed to bring a gene therapy drug to the European market, which is called 'scientific advice'. Investigators or sponsors may submit a description of the issue which they seek advice on and ask precise questions. These are first evaluated by the EMEA in order to avoid decisions on trivial issues that can be solved by consulting, for example, the *Rules Concerning Medicinal Products in the European Community*. Reasonable questions are then evaluated

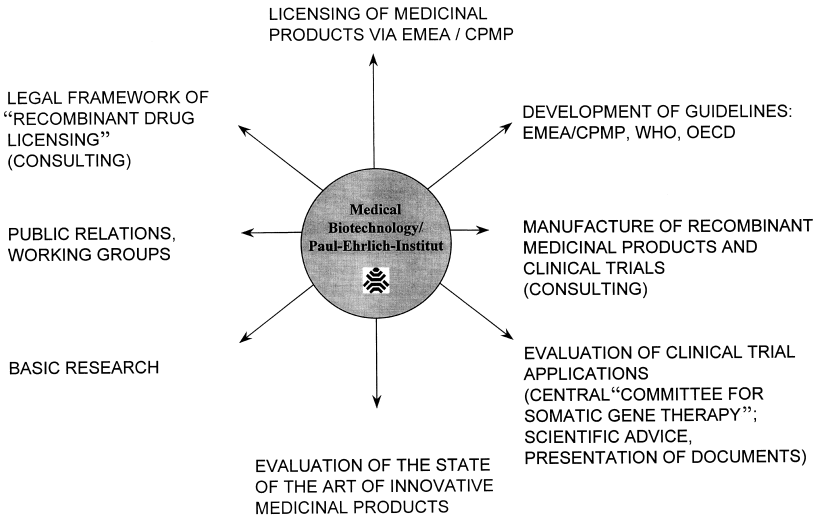


Figure 17.3. Involvement of the Paul-Ehrlich-Institut in gene therapy.

by a group of experts chosen by the coordinator of the specific scientific advice. A hearing with members of the party seeking advice and the coordinator's group of experts as well as interested CPMP members is then held in order to discuss the issues surrounding the questions submitted. A dossier containing the answers of the EMEA is subsequently discussed in the CPMP, and, if reviewed positively, forwarded to the sponsor or investigator who submitted the questions. This process may help to solve pertinent problems prior to the beginning of or during the clinical trials.

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18 The Transfer of Technology from the Laboratory to the Clinic: In Process Controls and Final Product Testing

FLAVIA BORELLINI and JEFFREY M. OSTROVE

18.1 INTRODUCTION

Gene therapy holds great promise as a treatment for a wide variety of genetic and acquired diseases. Since the initiation of the first human gene therapy trial in 1989, considerable progress has been made in the molecular engineering of both viral and non-viral gene delivery systems. More than 250 gene therapy protocols have been approved for clinical trial in the US, and over 300 worldwide. The use of potentially transmissible (replication-competent or replicating) vectors, as well as the possible applications of gene therapy to the treatment of non-life-threatening conditions, have brought new quality and safety issues to the attention of the scientific and regulatory communities. Successful growth in commercial gene therapy product development will require the joint effort of academia, industry and regulatory authorities in identifying and addressing areas of concern.

Among the many unique challenges encountered during development and clinical application of gene delivery systems, the design and implementation of appropriate and comprehensive quality assurance and safety testing programs is of utmost importance. Several regulatory authorities worldwide are actively involved in developing quality and safety guidelines for the production and testing of gene therapy products. This chapter provides a review of the recommended quality assurance and safety testing programs for different classes of gene therapy vectors currently in development or clinical trial.

18.2 REGULATORY OVERVIEW

Historically, a number of diverse problems related to contaminated products (simian virus 40 (SV40) contamination of the polio virus vaccine, hepatitis B contamination of a yellow fever vaccine, etc.) have led to the drafting and publication of regulatory guidelines covering safety testing of biopharmaceuticals.

With specific regard to the expanding field of gene therapy, the development of novel gene delivery systems and applications, and the increase in the number of protocols submitted for review, are unveiling new and diverse regulatory issues. As for any therapeutic product intended for human use, initiation of gene therapy clinical trials can take place only after various regulatory steps have been successfully completed and a number of scientific, manufacturing, and quality control issues have been addressed. In the US, production and safety testing of gene therapy products are regulated by the Food and Drug Administration (FDA) Center for Biologics Evaluation and Research. Numerous other committees, including local Institutional Review Boards and the Recombinant DNA Advisory Committee, may be involved in reviewing quality and safety data regarding the product and its manufacturing process. A number of 'Points to Consider' documents, the most recent in March 1998, have been published by the FDA to address various aspects related to quality assurance of gene therapy products, and to provide guidance to the scientific community and the biotechnology industry (Table 18.1). In order to foster development of new technologies, the FDA currently allows concurrent product manufacture and refinement, based on the application of a reiterative product development process.

In 1995, the European Union (EU) issued the Directive 'Gene therapy products – quality aspects in the production of vectors and genetically modified somatic cells,' and the Committee for Proprietary Medicinal Products (CPMP) is currently circulating a draft annex to address the safety issues of potential concern. In Member States of the EU, preclinical development and use of gene therapy vectors in clinical trials is reviewed in parallel by competent state authorities and advisory committees, which must approve the safety testing, production, clinical protocol, and ethical basis for therapy. Among such recently formed committees are the Gene Therapy Advisory Committee (GTAC), responsible for the oversight of gene therapy protocols within the United Kingdom, and the Commission for Somatic Gene Therapy in Germany.

Table 18.1 Regulatory documents relating to gene therapy

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- Points to Consider in the Production and Testing of New Drugs and Biologicals Produced by Recombinant DNA Technology 1985 US FDA (57 FR 33201)
 - Regulation of Gene Therapy in Europe: a Current Statement Including References to US Regulation 1994 (Cohen-Haguenauer, 1994)
 - Points to Consider in the Characterization of Cell Lines Used to Produce Biologicals 1993 US FDA (58 FR 42974)
 - Points to Consider on Plasmid DNA Vaccines for Preventive Infectious Disease Indications 1996 US FDA (61 FR 68269)
 - Points to Consider in Human Somatic Cell Therapy and Gene Therapy, 1991; update 1996 US FDA
 - Guidance for Industry: Guidance for Human Somatic Cell Therapy and Gene Therapy. 1988 US FDA
 - A Proposed Approach to the Regulation of Cellular and Tissue-Based Products 1997 US FDA (62 FR 9721)
 - ICH Draft Guidelines Q5A, Viral Safety Evaluation of Biotechnology Products Derived from Cell Lines of Human or Animal Origin
 - ICH Draft Guideline Q5D, Derivation and Characterization of Cell Substrates Used for the Production of Biotechnological/Biotechnology Products
 - ICH Draft Guideline Q6B, Specifications, Tests and Procedures for Biotechnological/Biological Products
 - ICH Draft Guideline S6, Preclinical Safety Evaluation of Biotechnology-Derived Pharmaceuticals
 - EC guideline III/5863/93, Gene therapy products – Quality aspects in the production of vectors and genetically modified somatic cells, 1995, and draft annex, 1998
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Note: US FDA documents can be obtained via the Internet at <http://www.fda.gov/cber/guidelines.htm>

18.3 GENERAL CONCEPTS OF QUALITY ASSURANCE AND SAFETY TESTING

Contamination of biopharmaceutical products with toxins, pyrogens, or adventitious microorganisms such as microbial and viral agents can cause both morbidity and mortality in the recipient. Before the treatment of patients with products of biological origin, it is extremely important to establish an appropriate quality assurance program to insure that these products are safe for human use (Ostrove, 1995; Smith *et al.*, 1996).

Like other biopharmaceuticals, gene therapy products need to be characterized with regard to safety, potency, identity, purity, and stability. However, due to the diverse nature of the products, the approaches to characterization of gene therapy vectors may present unique challenges, and require non-traditional approaches. For example, several procedures that have been shown to inactivate adventitious viruses, and are commonly built into the purification process of biopharmaceutical proteins, would destroy the ability of a viral gene therapy vector to transduce its genetic material into the host

cell. Thus, quality and safety have to be built into the gene therapy product, starting with the cell substrate used to produce the vector and the raw materials used for production. Stringent control of the production environment is also critical to preventing introduction of contaminants, as well as to maintain consistency in the properties and stability of the final product.

Building quality and safety into a gene therapy product includes definition of the methods and reagents used for production, as well as control of key intermediates such as cell banks. A well-conceived quality control and safety assurance program may involve a variety of simple procedures, such as cleaning and certifying biological safety cabinets at regular intervals, as well as complex studies, such as determination of biodistribution of the vector in animal models.

As a product moves from early development towards and through clinical trials, safety issues and regulatory requirements for product characterization may vary, based on changes in the vector, manufacturing scheme, and formulation. Regulatory agencies in the US sponsor a gradual approach to quality assurance and product characterization, to allow for continuous refinement of the product concurrently with preclinical and clinical evaluation. Thus, while limited product characterization and 'spirit of GLP' (good laboratory practice) practices are acceptable in the early stages of product development, full characterization of product using validated methods, development of product specifications, and full cGMP compliance are expected by phase III.

18.4 EARLY PRODUCT DEVELOPMENT

The early period of product development is critical in establishing a comprehensive program that addresses multiple aspects of quality and safety assurance, and in initiating a dialog with the appropriate regulatory agency.

To the purpose of identifying key quality and safety issues, and establishing appropriate controls for the production process, extensive characterization of the gene therapy construct should be performed at the early stages of product development. This may include characterization of source materials, documentation of vector construction and sequence, functional organization, and physical status of the vector. While for viral vectors of large size it may be sufficient to characterize and sequence the region containing the transgene and regulatory elements, complete sequencing should be performed in the case of plasmid DNA vectors. Whenever possible, safety features should be built into the vector design. For example, use of selection markers that confer resistance to antibiotics widely used in the clinic should be avoided in the design of plasmid-based vectors, to avoid the risk of spreading antibiotic resistance to environmental microorganisms.

In order to build safety into a gene therapy product, it is very important to understand how various contaminants could end up in the final product. To this purpose, in addition to characterizing the gene therapy vector, it is also necessary to qualify other components of the production and gene delivery system such as host cells, culture reagents, and formulation ingredients. The cell substrate used for vector production is a common source of contaminants. Since a significant amount of research effort goes into the development of substrate cell lines, when a few candidate cell lines have been identified, it is worthwhile to perform some preliminary safety testing to assure that no deleterious contaminants are present. The selection of appropriate assays will depend on the nature of the cell line and the vector produced, and while some tests are generally applicable to a variety of gene delivery systems, others are specific to a type of vector or producer cell line.

In the case of mammalian cell substrates, the preliminary testing of raw materials used in culture allows for detection of contaminants before significant investment of time and resources. Bovine serum, used as an additive to cell culture media, and trypsin, used for cell detachment, have been shown to harbor viral and microbial contaminants, and should be screened for the presence of infectious bovine and porcine viruses. In these *in vitro* assays, indicator cell lines such as porcine testicle (PT-1) or bovine turbinate (BT) cells are inoculated with the test material and observed for cytopathic effects (CPE) at regular intervals over 14–28 days. If other components are introduced during manipulation of culture conditions, such as cytokines, antibodies, or solid support beads, specifications and acceptance criteria for these materials should be established.

Since most research cell lines are routinely grown in antibiotic-containing medium, candidate cells for the production of viral vectors should be tested for sterility and mycoplasma, after 2–3 culture passages in the absence of antibiotics. To maximize the range of detection to contaminants with different growth requirements, sterility and mycoplasma assays should be conducted using multiple broth and agar media, and should employ aerobic as well as anaerobic conditions. In addition, Sabouraud dextrose agar should be used for the detection of fungal contaminants. Mycoplasma should also be detected by direct inoculation of the test cells onto an indicator cell line such as Vero African green monkey kidney cells. The indicator cells should then be stained using Hoechst stain, a DNA fluorochrome that allows visualization of mycoplasma under fluorescent light.

The preliminary screen of cell lines used to produce retroviral vectors should include a test to detect the presence of replication-competent retroviruses (RCRs), such as the sensitive S + L – focus assay (Haapala *et al.*, 1985; Hughes *et al.*, 1996). In this assay, feline PG-4 cells that contain a replication-defective sarcoma virus genome are used as indicator cells. In the presence of

RCR serving as helper virus, PG-4 cells exhibit a transformed phenotype, and the formation of 'foci' of transformed cells is visualized by low power light microscopy

In addition to screening of the substrate cells, development of adenovirus, poxvirus, and herpes simplex virus (HSV) gene therapy systems requires preliminary characterization of the viral seed stock, to include sterility and mycoplasma tests. These tests should also be performed on seed stocks of viruses used as helper for the production of adeno-associated virus (AAV) vectors. When the viral vector is a replication-defective adenovirus, preliminary screening of the seed stock should also include an *in vitro* assay for the presence of replication-competent adenovirus (RCA). The RCA assay should utilize an indicator cell line, such as the human lung carcinoma A549, which is permissive to infection by replicative adenovirus.

In the case of prokaryotic cells used for the production of plasmid-based vectors, testing for bacteriophage should be considered, since its presence could affect stability of the vector/host cell system and product yield.

If satisfactory results are obtained on preliminary screening, master cell bank (MCB) and master virus bank (MVB) can then be established. Table 18.2 outlines the recommended steps for prescreening of candidate cell lines and establishment of mammalian cell banks.

18.5 ESTABLISHMENT AND TESTING OF MASTER CELL BANKS AND MANUFACTURER'S WORKING CELL BANKS

Once a candidate cell substrate (for example 293 or perC6 cells for adenovirus production, or PA317, PG13, AM12 for growing retroviruses) has been identified, and shown to produce adequate yields of genetically and biochemically stable vector, it is necessary to establish cell banks according to a two-tiered system comprising master and working cell banks (Steuer and Ostrove, 1996). While the MCB is a collection of cells of uniform composition derived from a single tissue or cell, the manufacturer's working cell bank (MWCB) is a uniform collection of cells derived from one or more vials of the MCB.

The establishment of cell banks represents a significant part of the transition from a research program to a manufacturing program. This process is an integral part of manufacturing and should be performed according to current Good Manufacturing Practices (cGMP), as defined in the US Code of Federal Regulations (21 CFR), the *Rules Governing Medicinal Products in the European Community*, Good Manufacturing Practice for Medicinal Products (Commission Directive 91/356/EEC, 1991), as well as in numerous other regulatory documents from other countries such as Japan, Australia, and Canada. Table

Table 18.2 Establishment of mammalian cell banks under cGMP

-
1. Receive cell in quarantine
 2. Place cells in culture in an established 'clean' environment for preliminary testing
 3. Test for mycoplasma, sterility, and additional system-specific tests (for example RCR test)
 4. Release cell lines that passed prescreening, and transfer into a GMP suite (validated equipment, procedures, and documentation)
 5. Expand cells to desired numbers, mix to create a uniform pool, aliquot, and cryopreserve in liquid nitrogen (routinely 100 vials at 10^7 cells in 1 ml vials)
 6. Following a minimum of 72 hours, thaw aliquots (3–5% of the bank) and test viability and plating efficiency
 7. Expand cells and prepare samples for safety testing according to the various regulatory documents (Table 18.1)
 8. Release MCB upon certification and place into a quality controlled environment (still following cGMPs)
 9. Generate a cell bank report and Certificate of Analysis that can be used for regulatory submissions
 10. Perform additional post-bank characterization tests for activity, titer, etc.
 11. Upon completion of characterization tests, establish MWCB using similar cell growth procedures. The size of the MWCB will vary according to manufacturing needs
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18.2 outlines the suggested steps towards the establishment of mammalian cell banks under cGMP practices.

As for all biopharmaceuticals, the cell substrate used in the manufacturing process should be thoroughly characterized to demonstrate that the cell identity is as expected, and that the cells are not harboring adventitious agents. As many cell types have been used as substrates for the production of different gene therapy vectors, a comprehensive list of all cell types and appropriate tests is not feasible. Description of the type of tests that need to be performed on different cell substrates can be found in several regulatory documents (Table 18.1). Since the majority of approved clinical trials worldwide still use engineered viruses to transduce genetic information into target cells, we will concentrate on the cell substrates most frequently used in the production of viral vectors. Retroviruses are commonly produced on murine (3T3-derived) cells, while adenoviruses and AAV vectors are produced on cells of human origin (often 293 cells, derived from human embryonic kidney). Clinical protocols employing retroviral vectors produced in canine cells (Jolly, 1995), as well as oncolytic replicating herpesviruses produced on Vero African green monkey kidney cells (Ostrove, unpublished), have also received regulatory approval and have entered clinical trials.

For murine and human cell substrates, tests for sterility, mycoplasma, and *in vitro* and *in vivo* tests for detection of adventitious viral contaminants should be performed on the MCB as well as the MWCB (Table 18.3). Several

Table 18.3 General characterization and safety testing of MCB and MWCB

-
- Sterility
 - Mycoplasma
 - Isoenzymes for identity confirmation
 - *In Vitro* co-cultivation assay for adventitious viral contaminants
 - *In Vivo* assay for adventitious viral contaminants
-

Table 18.4 Specific characterization and safety testing of murine and human MCB

-
1. *Murine MCB*
 - Extended XC plaque assay for ecotropic retroviruses
 - Supernatant: detection of retrovirus by amplification in *Mus dunni* cells^a and detection on PG-4 cells (5% of production lot volume is required for testing)
 - Producer cell testing: detection of retrovirus by co-cultivation with *Mus dunni* cells^a and detection on PG4 cells (1% of the total producer cells, pooled or 10⁸ cells are tested, whichever is less)
 - Mouse antibody production (MAP) for the detection of 16 different pathogens that infect murine cells
 2. *Human MCB*
 - Transmission EM for viruses
 - PBL co-cultivation for detection of human immunodeficiency virus-1, -2
 - Detection of human viruses (Epstein-Barr virus, cytomegalovirus, hepatitis B, human T-cell lymphotropic virus-1, -2, Hepatitis C, human herpes virus 6 and 7) by PCR
 - *In Vitro* tumorigenicity, soft agarose
 - Cytogenetics
-

^aIf the retroviral vector envelope is derived from the gibbon ape leukemia virus (GaLV) the amplification and co-cultivation assays should be performed in 293 cells rather than in *Mus dunni* cells.

other tests, designed to address concerns specific to a cell substrate, need only be performed on the MCB (Table 18.4). For example, in the case of murine cell substrates used for the production of retroviral vectors, testing of the producer cell line (packaging cell line with retroviral vector containing the specific gene of interest) should involve both producer cell and supernatant testing and include assays for murine retroviruses and RCR (Kahn, 1996). Testing of the human cell substrates used in the production of adenoviral vectors should include assays for specific human viruses, AAV, as well as assays for cell identification and *in vitro* tumorigenicity (Table 18.4).

Characterization of prokaryotic cell banks should also be mentioned, as several protocols involving the use of plasmid DNA produced in bacterial cells are currently in clinical trial. Cell identity (by genotype and phenotype determination), absence of bacteriophage as well as stability of the vector/host system should be evaluated. In particular, evaluation of the vector/host

system should include determination of plasmid copy number per host cell, plasmid retention, and vector stability (by restriction mapping or polymerase chain reaction, PCR).

Once the MCBs have been fully characterized and shown to be free of adventitious agents, the cell substrate can be used for manufacturing of clinical product. All manufacturing should be conducted following cGMP principles, and to ensure the quality and safety of the final product, raw materials such as serum, trypsin, and any chemical or biological additive should be acquired from a cGMP-compliant source. A Certificate of Analysis for each of the raw materials used in the manufacturing process should be obtained, examined, and filed along with the production records.

The cGMP laboratory should have controls designed to produce a uniform sterile product, including appropriate certification and validation of all equipment used in manufacturing, and proper documentation and training of the production staff. Air handling equipment should meet Class 100 (less than 100 particles $0.5\ \mu\text{m}$ in size per cubic foot of air) in all locations where open vessel procedures are performed. Detailed production records (Batch Records), which document all steps in the production process and all materials used, should be generated, to allow for re-evaluation of all manufacturing steps if a problem arises following production or during clinical trials.

18.6 TESTING OF THE MASTER VIRUS BANK OR MASTER SEED STOCK

Production of retroviruses usually involves the use of cell lines that are constitutively budding virus into the cell culture media, and the cell substrate is used directly for manufacturing. In the production of other viral vectors such as adenovirus, AAV, poxvirus and herpesvirus, the cell substrate is expanded in culture and then infected (and/or transfected) with the vector to be manufactured. In this case the virus used in the manufacturing process needs to be qualified and shown to be free of adventitious agents. A master virus bank (MVB) and if necessary a manufacturer's working virus bank (MWVB) are created following cGMP guidelines, and using the previously qualified MCB or MWCB as substrate. As an example, Table 18.5 illustrates testing involved in the generation of an adenovirus MVB. The cell harvest used for production of the virus bank is tested for sterility, mycoplasma, adventitious viral contaminants, and AAV. The MVB is tested for sterility, mycoplasma, and the presence of RCA by blind passages on A549 cells. In the case of plasmid vectors, the master seed stock needs to be characterized in terms of identity and structure by a variety of approaches such as sequencing, restriction mapping, and PCR analysis.

Table 18.5 Characterization and safety testing of master virus bank (MVB)

1. <i>Cell harvest</i>
• Sterility
• Mycoplasma
• <i>In Vitro</i> assay for adventitious viral contaminants
• <i>In Vivo</i> assay for adventitious viral contaminants
• Detection of human viruses by PCR (EBV, CMV, Hepatitis B, HIV-1, -2, HTLV-1, -2, AAV, parvovirus B-19)
• Additional human pathogens such as HCV, HHV6, and HHV7 can also be tested by PCR
2. <i>Master virus bank</i>
• Sterility
• <i>In vitro</i> assay for the presence of replication-competent adenovirus (RCA)
• Titer

18.7 TESTING OF CLINICAL PRODUCTS

The first gene therapy protocols utilized replication-defective murine retroviruses to transduce foreign genes. Since generation of RCR might result from recombination between vector sequences and sequences in the packaging line, numerous studies were performed to demonstrate the safety of murine amphotropic retroviruses in animal models including non-human primates (Cornetta *et al.*, 1990, 1991a,b). These studies showed that these viruses were not acute pathogens and did not cause viremia in non-human primates. However, in 1992 Donahue *et al.* reported the development of T cell lymphoma in immunosuppressed non-human primates receiving RCR-transduced CD34+ cells, and characterization of RCRs indicated that they had originated by recombination between vector and packaging encoding sequences in the producer cell substrate (Vanin *et al.*, 1994). As a consequence, the US FDA announced a more stringent testing program for both the cells and supernatant produced during the manufacturing of defective retroviruses for human clinical trials (Gunther *et al.*, 1993; FDA Vaccines and Related Biological Products Advisory Committee Meeting, October, 1993; Ostrove, 1995). In general, these testing programs are still followed today and current recommendations are shown in Table 18.6 (Wilson *et al.*, 1997). Testing of the supernatant preparation and end of production cells include assays for microbial contaminants, endotoxins, extraneous toxins, and RCR. RCR testing of the supernatant presents an additional challenge, since the recombinant retrovirus interferes with the sensitivity of detection of standard culture assays (Printz *et al.*, 1995). To overcome the interference and enhance the sensitivity of the S + L – focus assay, RCRs can be first amplified in culture by inoculation of *Mus dunni* cells (Hughes *et al.*, 1996; Lander and Chattopadhyay, 1984). Following amplification, supernatant from the cul-

Table 18.6 Testing of retroviral vector supernatant and end-of-production cells

-
1. *Unprocessed bulk testing*
 - Sterility
 - *In vitro* assay for adventitious viral contaminants (FDA/CBER requires this assay for phase III clinical trials only).
 2. *End-of-production cells*
 - Sterility
 - Co-cultivation with *Mus dunni* cells^a (1% of the total producer cells, pooled, or 10⁸ cells, whichever is less).
 3. *End-of-production cells/unprocessed bulk*
 - Mycoplasma
 4. *Filtered bulk*
 - Detection of retrovirus by amplification in *Mus dunni* cells^a (5% of production lot volume is required for testing).
 5. *Final container*
 - Bacteriostasis/fungistasis to demonstrate if the final product or its formulation inhibits the sterility assay (only performed once per formulation).
 - Sterility
 - General safety
 - Endotoxin (limulus amebocyte lysate)
-

^aIf the retroviral vector envelope is derived from the gibbon ape leukemia virus (GaLV) the amplification and co-cultivation assays should be performed in 293 cells.

ture is placed on PG-4 cells and the cells are observed for focus formation. Other endpoint assays, including the marker rescue assays described by Miller and Buttimore (1986) and Forestell *et al.* (1996), are acceptable to regulatory authorities if validated.

Adenovirus, AAV, and herpesvirus are all produced in primate cells, and virus is harvested at a certain time point following infection or transfection. The viral vector can be found either inside the producer cells, or distributed between supernatant and intracellular compartments. Therefore, samples for testing are taken from either the cells or cell and supernatant, in order to achieve the highest likelihood of finding adventitious agents. Testing of clinical lots is performed on the cell harvest and also on the final vial product. Regardless of the type of vector, testing of the cell harvest should always include assays for adventitious microbial and viral contaminants (Table 18.7). Additional tests, specific to the gene delivery system, may also be required. For example, cell harvest from adenovirus production should be tested for the presence of AAV. Tests on each final production lot should include bacteriostasis/fungistasis, sterility, general safety, endotoxin, and virus titer (Table 18.8). Based on the nature of the vector, additional tests may be required.

Testing of final product varies significantly for plasmid vectors, and in addition to sterility, general safety, and presence of endotoxin should address the presence of protein, RNA, residual host DNA, and ratio of lin-

Table 18.7 Testing of adenovirus, AAV and herpesvirus vector cell harvest

-
1. *General tests*
 - Sterility
 - Mycoplasma
 - *In vitro* assay for adventitious viral contaminants
 2. *Vector-specific tests*
 - PCR assay for detection of AAV (Adenovirus vectors)
 - Reverse transcriptase (HSV)
 - *In vivo* assay for adventitious viral contaminants
-

Table 18.8 Testing of adenovirus, AAV and herpesvirus final product

-
1. *General tests*
 - Sterility
 - Mycoplasma
 - General safety
 - Endotoxin
 2. *Vector-specific tests*
 - Particle count by OD₂₆₀ (adenovirus vectors)
 - Protein concentration (adenovirus vectors)
 - *In vitro* RCA assay (adenovirus vectors)
 - *In vitro* assay for the presence of wild-type AAV (AAV vectors)
 - Assay for the presence of helper virus (adenovirus or herpesvirus) if used (AAV vectors)
-

ear: supercoiled vector. Moreover, if antibiotic or other chemicals (lipids, local anesthetics) were used for production or formulation, residual amounts should be quantitated in the final product. If toxic substances, such as organic solvents, were used for production or formulation, lot release specifications should include specific testing to demonstrate their removal.

18.8 PRECLINICAL TOXICOLOGY AND PHARMACOKINETIC STUDIES

In a Guidance for Industry document (Guidance for Human Somatic Cell Therapy and Gene Therapy, 1988) the US FDA addresses preclinical toxicology and pharmacokinetics studies for gene therapy vectors. Generally, these studies should be conducted using the final formulated product, and address various issues for labeling including genotoxicity, mutagenicity, toxicity, and carcinogenicity, as well as effects on reproduction and development. Additionally, a well-conceived preclinical toxicology study should help define critical parameters for use in clinical trials, such as safe starting dose and escalation scheme, potential organ-specific toxicity, appropriate

means of clinical monitoring, and inclusion/exclusion criteria for patient population.

While not always necessary, *in vivo* models are preferable when determining the safety of novel therapies, or when previous adverse findings have been reported with a similar class of vector. However, gene therapy vectors present unique scientific and safety issues that standard animal toxicology studies cannot adequately address. The selection of an animal model should carefully consider relevance to the vector biology and to the intended route, dose and regimen of delivery, availability of disease models, immunogenicity (which in some instances is the desired effect), intended patient population, and potential patient benefits. In addition, age and sex of animals are variables to be carefully considered. The selected route of administration should be as close as possible to the anticipated route in humans. An additional study with intravenous administration may be performed as a 'worst possible case scenario,' to evaluate the effects of maximum organ exposure. Doses for treatment should range from no-effect to overtly toxic, and include equivalent as well as multiples of intended human dose (scaled to body weight or surface area), with the aim of identifying adequate safety margins. Selection of regimen (single versus repeated administration, acute versus chronic, real time versus accelerated schedule) is also critical, and should be designed with the intent to uncover potential toxicity, as well as with consideration to the intended use.

Data collected from *in vivo* preclinical studies should include traditional toxicology endpoints such as daily clinical observation, clinical chemistry, hematology, histopathology, and immune response. In addition, other issues such as vector dissemination, expression, clearance, and integration in the host genome should be addressed on a case by case basis, dependent on the vector biology and intended patient population, and interpreted with consideration to the host immune response. For example, if the vector appears to persist in the gonads, it may be advisable to attempt to identify the type of cell transduced, and the possibility of vertical transmission by vector integration into the germline, especially if the target patient population comprises individuals with reproductive potential (Doerfler *et al.*, 1997).

With regard to carcinogenicity, the classic chronic rodent bioassay is clearly not appropriate if the product is immunogenic. In this case, *in vitro* assays to assess growth promotion in relevant cell lines may be more appropriate. Genotoxicity can also be assessed *in vitro* using human cell lines.

18.9 CONCLUSIONS

Establishment of a comprehensive quality assurance and safety testing program is clearly critical to the development of safe and effective gene delivery

systems. The design of preclinical quality assurance and safety studies should be science-driven, and should aim at answering specific questions using the best available science and technology. In any event, it is very important to remember that even the best-designed preclinical program may have pitfalls, especially with regard to toxicity. For example, reversibility of toxicity may be missed, adverse effect observed in animals may not be relevant to humans, and long-term toxicity may not become observable through the duration of the study. Ultimately, only the widespread application for human use will provide an accurate safety assessment.

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19 Good Laboratory Practice in the Research and Development Laboratory

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19.1 INTRODUCTION

The paramount consideration in the design and manufacture of an agent for medicinal use is to ensure that the production process is both safe and controlled. Two codes of practice have been developed to ensure conformity to these requirements: Good Manufacturing Practice (GMP) and Good Laboratory Practice (GLP). The basic principles of these codes of practice are discussed briefly below.

19.2 GOOD MANUFACTURING PRACTICE

In the UK Medicines Control Agency's *Rules and Guidance for Pharmaceutical Manufacturers* (1993) it is stated that the majority of reported defective medicinal products resulted from human error or carelessness. In order to prevent or limit the occurrence of such errors, a system of controls with emphasis on hierarchical testing and documentation (GMP) has been developed for the manufacture of medicinal products, including biologics. The main components of GMP comprise controls of both production and quality. Production control is concerned with manufacturing, including the suitability of the facility and staff for manufacture development of standard operating procedures (SOPs) and record keeping. Quality control is concerned with sampling, specifications and testing, and with documentation and release procedures, ensuring satisfactory quality.

19.3 GOOD LABORATORY PRACTICE

The testing referred to above is conducted according to Good Laboratory Practice (GLP), which assures the quality and validity of the data generated (Department of Health, 1989, 1997; Food and Drug Administration, 1997). GLP is regulated by the Department of Health in the UK, where it is defined as 'the organisational processes and the conditions under which laboratory studies are planned, performed, monitored, recorded and reported' (Department of Health, 1989).

Compliance with GMP and GLP guidelines requires monitoring by defined quality assurance staff operating independently of personnel involved in manufacturing or testing. Clearly the implementation of such standards for early phase clinical trial material is a costly procedure. This is of concern to many academic groups, which are being funded to conduct time consuming research into gene therapy delivery systems for use in clinical protocols. There is a necessity to ensure that potential therapies based on viral vector gene delivery systems enter clinical trials in a timely and cost effective manner without, however, compromising the safety and quality of the agent administered. This is a difficult balance to achieve as the control and safety of the cell lines and of the production process for viral vector stocks is paramount for a product that is subjected to, at best, minimal purification.

Compliance with GMP and GLP standards is the optimum for production of a medicinal product and these standards should therefore be applied whenever possible. The investment in terms of time and cost in attaining such quality standards and safety controls in a clinical protocol is a wise long-term strategy, which could prevent unforeseen contamination of a novel gene therapy agent (Smith *et al.*, 1996). While it is evident that GMP applies to the industrial production process and GLP to testing associated with the process, the manner in which the product may have been developed initially has historically been less controlled. Biopharmaceutical products may begin development either in academic or industrial research laboratories, where requirements to comply with such regulations are lacking. GLP regulations in both the European Union (EU) and the USA pertain specifically to non-clinical laboratory studies to determine the safety of test substances (Department of Health, 1997; Food and Drug Administration, 1997). However, there is recognition in industry, accompanied by increasing pressure from regulatory bodies, that processes should be controlled at as early a stage in development as possible. In academia, also, there is heightened awareness of the need for research and development to be conducted to standards that will be acceptable to retrospective regulatory scrutiny. While simple adoption of full GLP compliance for such studies would be the ideal, there are a number of factors which render such a course difficult for many laboratories, the most important of which is financial. GLP requires an independent quality assur-

ance unit, the prime responsibility of which is to ensure that studies are performed in accordance with GLP principles. Provision of the specific personnel and resources, including time, which require to be allotted to this function is problematic for academic laboratories in particular.

Thus, while adoption of the principles of GLP remains a standard which may not be applicable to research and development, it is nevertheless possible and desirable to incorporate many of the tenets of GLP into early development programmes. The principal features of GLP which should be adopted into the research and development laboratory are: (i) traceability of reagents and materials, (ii) adherence to written protocols (SOPs) and study protocols (SPs) where possible, and (iii) designation of personnel (study directors) with responsibility for defined aspects of studies. This chapter sets out the principal considerations in order to achieve standards of quality assurance in accordance with the principles of GLP for each of the above aspects. In addition, it seeks to address the role and function of quality assurance in relation to research and development.

19.4 RESPONSIBILITY

The overall responsibility for ensuring that the principles of GLP are complied with for any facility, industrial or academic, rests with the designated management (Department of Health, 1997). The principal duties of management are to ensure that appropriate personnel, facilities, equipment and materials are available, that appropriate documentation is implemented and archived and that there is a quality assurance programme with designated personnel. In addition, management should designate an appropriately qualified and trained individual as study director.

19.5 TRACEABILITY

It is important that all reagents and materials used during research and development are of optimal quality and free from contamination. Materials should be obtained from sources where documentation exists regarding the acceptability of the reagent in relation to defined criteria. Documentary verification of the specifications of most materials and reagents is provided by the manufacturer in the form of a certificate of analysis (C of A), which will apply to each lot or batch.

For cell cultures, important aspects to be documented include source, passage history and manipulations, media (including raw materials of animal origin), potential exposure to contaminating agents and the degree of

testing to determine freedom from contamination. Where possible, cell lines, antibodies or infectious agents should be obtained from recognised repositories such as the American Type Culture Collection (ATCC) or the European Collection of Animal Cell Cultures (ECACC). For primary cell cultures derived from humans, it is important that information on the health status of the donor is obtained, particularly with regard to infectious viruses. Similar considerations should apply for primary cultures derived from animals. Where necessary, additional testing of cell cultures or virus stocks should be performed to ensure freedom from contamination with agents which could compromise the safety of operators or the product.

Raw materials of animal origin which may be used in cell culture include foetal bovine serum and porcine trypsin, both of which may be contaminated with viruses (McLean *et al.*, 1997). Manufacturers' C of As for such materials should contain results of assays for viral contamination in addition to other specifications. It is important that bovine serum is sourced from countries without endemic bovine spongiform encephalopathy (BSE). Reagents of human origin (for example those derived from human blood) should be accompanied by adequate information regarding the health status of the donors. Supplementary testing should be performed where appropriate.

For key raw materials, documentation received should be verified by audit investigation to confirm that the information provided accurately represents the supplier's procedures and that the process for certification is adequate. Such an audit should be performed before use of the material. The results of the audit should be used not only as the basis for acceptability, but also to determine whether supplementary testing of the material should be performed.

Each batch, lot or individual item used in research and development should be allotted a unique material receipt, or receiving number, and should be labelled accordingly. Labels should also record information such as storage conditions and expiry dates.

It is important to consider the environment in which development and manipulations are performed. Other work being performed in the same laboratory may present the possibility of cross-contamination of cell lines or of contamination with infectious agents. Particular attention should be given to storage of reagents, working samples and stocks in order to avoid cross-contamination. Storage of materials in liquid nitrogen Dewars, freezers and fridges should be adequately documented.

19.6 DOCUMENTATION

Good laboratory practice in the collection and handling of raw data is as important in the development phases of biopharmaceutical products as in

the later stages of manufacture. In order to conform to GLP, a programme of work in the laboratory should proceed according to a written study plan. The study plan should define the objectives, rationale and methods of the study to be performed. In addition, the study plan should designate the individual principally responsible for the study (the study director). For research and development studies, where procedures may require change dependent on results, the study plan should be structured in such a manner as to retain flexibility.

Procedures, where possible, should follow written study protocols. Such protocols should record data such as start and finish times of assays, the materials and equipment used, the procedures followed and the results obtained. This will be justified where certain assays or procedures are performed repeatedly throughout the study. Procedures which are performed once only, infrequently or variably are more appropriately described and the raw data recorded in laboratory notebooks. Such notebooks should be issued for specific studies with the names and signatures of the investigators and study director.

The functions of the laboratory should be regulated by SOPs, written procedures for policy relating to all aspects of work, which should be adhered to by relevant personnel. SOPs should detail the procedures for all aspects of the working environment, including material receipt and storage, disinfection procedures, equipment use, calibration, monitoring and maintenance, appointment of study directors, documentation control, scheduling of work, and report writing and archiving.

19.7 THE ROLE OF THE STUDY DIRECTOR

The role of the study director is critical to the conduct of routine or developmental studies to GLP (Department of Health, 1992). As noted above, the study director is the individual designated by management who has responsibility for the planning, implementation and reporting of studies. It is the responsibility of the study director to oversee the elements of the work with the plan and to ensure the accuracy and validity of the studies performed alone or by others. It is also the responsibility of the study director to prepare a final report which accurately reflects the raw data generated during the study. The principal duties of the study director are:

- to ensure that adequate resources, documentation in the form of SOPs, where suitable, and suitably trained personnel are available to perform the study prior to commencement.
- to approve the protocol by dated signature.
- to ensure appropriate liaison with and awareness of the protocol in all key staff involved (including quality assurance) in the study.

- to be responsible overall for the technical performance of the study. This will involve continuing review of the study procedures.
- to ensure that procedures are being followed accurately. The study director should be aware of the progress of the study by maintaining effective communication with scientific, administrative and quality assurance personnel.
- to ensure that the raw data generated are fully and accurately documented and that deviations or amendments to the protocol are accurately reflected.
- to produce a detailed scientific report which accurately reflects the raw data obtained in the study.

After audit by quality assurance the study director should sign and date a statement to the effect that the study was performed according to the principles of GLP.

19.8 THE ROLE OF QUALITY ASSURANCE

In order to implement an effective quality assurance programme, the quality assurance unit must be independent of the personnel who perform research and development studies. In certain facilities it may not be practicable to maintain personnel dedicated solely to quality assurance. In such cases, one individual may be appointed to have permanent, but part-time, responsibility for the quality assurance function. In general, the functions of quality assurance in relation to research and development are identical to those for non-clinical safety studies. The principal responsibilities of quality assurance are:

- to maintain a master schedule listing of all studies planned, current or completed.
- to review study plans before commencement of studies in order to ensure the completeness and compliance of the protocol with GLP principles, and to identify appropriate phases of the study for monitoring.
- to regularly monitor facility operations, periodically review studies and documents, schedule and keep appropriate records of audits and inspections of all studies, and report the findings of such reviews and inspections to management.
- to review facility SOPs before issue in order to ensure clarity, control the production and issue of SOPs, and ensure that SOPs are periodically reviewed by appropriate personnel.
- to audit raw data either during inspections or by audit of final reports.

19.9 SUMMARY

Although the GLP regulations pertain specifically to non-clinical laboratory safety studies, they can be readily applied to experimental research and development. The implementation of such standards in the research and development of biological agents for gene therapy would enhance considerably the existing measures employed to ensure product safety.

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20 Facilities for Large-Scale Production of Vectors under GMP Conditions

JANICE E. BOYD

20.1 INTRODUCTION

At present, the vectors used in gene therapy are of two major types – virus-based, such as retrovirus, adenovirus or herpesvirus, and plasmid DNA in the form of ‘naked’ DNA or liposomes. Thus, two types of culture system are required for vector production: (i) mammalian cell culture coupled with currently limited downstream processing and (ii) bacterial fermentation, which is associated with more complex downstream processing. Since gene therapy is still in its early stages of development, it is unlikely that a manufacturing facility would be set up for a single product only, especially since few gene therapies are in phase III trials yet. In addition, the current scale of manufacture is relatively small compared with conventional pharmaceuticals. Nevertheless, similar culture and fermentation systems can be used for a multitude of different products. This situation lends itself to the development of multi-product facilities, which offer savings in terms of cost and time, since manufacture can be switched from product to product relatively easily.

This chapter discusses many of the issues that must be considered in the design and running of a production facility. There are many regulatory guidelines that have to be interpreted so that compliance is accomplished, but the overall outcome must be a safe and efficacious product. This is no different from any other pharmaceutical product, but the means of achieving it are, because of the nature of the product. Batches of chemically-derived products are relatively easy to control since there are physical means to measure their purity and activity. Biologically-derived products are much more difficult to control on a batch basis since often there is no easy method for measuring purity or activity. In this case, much more emphasis is laid on

control of the manufacturing process itself, including the starting materials, what measures are taken to prevent contamination by unwanted organisms, and validation that all procedures are effective.

20.2 GOOD MANUFACTURING PRACTICE

20.2.1 GUIDELINES

Good manufacturing practice (GMP) is only one part of the quality assurance (QA) system that must be in place to ensure that a medicinal product is of the quality required for its intended use. All the relevant issues as they relate to the European Union (EU) are outlined in the 1997 update of the 'Orange Guide' [1], and the salient points are presented in Tables 20.1–20.3.

QA is a wide-ranging concept that incorporates GMP and other aspects of quality management. GMP is that part of QA that ensures that products are consistently manufactured and controlled, while quality control (QC) is the part of GMP concerned with sampling, specifications and testing, and final release of the product.

Quality management, as outlined above, is only the start of a manufacturer's responsibilities, and the Orange Guide covers all aspects, including personnel, premises and equipment, documentation, production, quality control, contract manufacture and analysis, complaints and product recall, and self-inspection. Once a system is in place, it will be inspected by the appropriate authority – in the UK by the Medicines Control Agency – which, when satisfied, will issue a Certificate of Compliance. However, working to GMP is a dynamic situation and the system must be monitored by regular self-inspection and updated using controlled methods.

Personnel are the key to the effective operation of a GMP system. Job descriptions must be written and each individual's responsibilities clearly defined and understood. An organisational chart must be drawn up to show reporting relationships and responsibilities. There must be separate individuals in charge of production and quality control.

The individual in charge of quality control will also approve specifications, such as for materials and products, and will have the authority to pass or reject any material, including the final product.

The production manager has control over all aspects of production, including procedures, equipment and records, and is responsible for ensuring that the correct manufacturing procedures are followed using the appropriate documentation.

In addition to their individual responsibilities, these key personnel are jointly responsible for authorising written procedures, monitoring and main-

Table 20.1 Quality assurance

<ul style="list-style-type: none">• Product is of the quality required for its intended use• Production and control operations are clearly defined• Managerial responsibilities are clearly defined• Correct starting and packaging materials are used• In-process controls and validation are carried out• Finished product is correctly processed• Storage and handling do not compromise the product• Self-inspection and audit procedures are in place

Table 20.2 Quality control

<ul style="list-style-type: none">• Sampling, inspection and testing of materials• Validation of test methods• Finished product meets specification• Review of documentation• Reference samples of starting materials and product retained• Batch release controlled by Qualified Person

Table 20.3 GMP

<ul style="list-style-type: none">• Part of Quality Assurance system• Manufacturing process clearly defined• Critical steps are validated• Only approved procedures and instructions followed• Deviation and change control of the process• Batch records allow full traceability
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tenance of the production areas, personnel training and approval of materials suppliers.

20.2.2 PRACTICAL CONSIDERATIONS

In addition to the Orange Guide, there are a number of European Directives and other documents which cover the contained use of genetically modified organisms [2] and recombinants [3–6]. These deal with some of the practical issues but with particular emphasis on in-process control of the production procedure so as to ensure consistency of the final product. This can be achieved by starting with quality reagents, including the culture or fermentation media, monitoring the production organisms for genetic drift, examining the effects of ‘scale-up’ on the product, and controlling cell banks to avoid cross-contamination.

How this can be accomplished practically is outlined in Table 20.4. Only approved suppliers should be used, following a satisfactory audit of their

Table 20.4 Practical considerations of GMP

-
- Only approved suppliers used following satisfactory audit
 - C of As for all chemical components
 - Origin and testing of all biological components
 - Sterility certificates for plastic disposables
 - Full documentation: batch numbers, receiving numbers, storage control
 - Equipment validation and monitoring
-

facilities and procedures. Full GMP compliance requires that all incoming raw materials are batch tested for identity, purity etc. However, for facilities producing material for early clinical trials it is usually sufficient that a satisfactory supplier audit has been performed. In the case of sterile materials such as media and supplements, it is prudent to build up a history of test results to confirm the supplier's own tests. Thus, for each new sterile liquid material received, the first three batches would be re-tested for sterility and mycoplasma or other appropriate parameters, then if these were satisfactory the supplier's results could be accepted for future batches of that material.

Chemicals and biologicals must be supplied with Certificates of Analysis for each batch as well as Certificates of Origin for biologicals such as animal sera. In addition, for animal sera an audit trail should be supplied which identifies the origin of the sub-lots that were pooled to make that supplier's batch. As a consequence of the supplier's audit, it may be felt that certain tests should be supplemented. Thus, for example, we perform extra virus testing on biologicals such as foetal calf serum and trypsin in an attempt to identify viruses that may not have been detected by the supplier's tests.

For sterile materials such as plastic disposables Certificates of Sterility or Certificates of Irradiation should be obtained.

In all cases, there must be documentation procedures that allow traceability of the use of any starting material. This can be achieved by the application of a unique number to each incoming batch of raw material. The record should contain information such as the supplier's batch number and expiry date, storage conditions and location, confirmation that appropriate documentation has been received, and the results of any testing performed. Finally, the material should be passed for manufacturing use or rejected, as appropriate. Likewise, the container of the raw material must be appropriately labelled with its unique number and an indication of its current status, such as Quarantine, On Test or Passed. Separate storage facilities are required for materials that are in quarantine, passed or rejected. Once passed, then accurate stock records must be maintained to document the history of the material's use, and any expired material must be disposed of in a controlled manner.

When you multiply this procedure by the number of raw materials and goods received by even a small manufacturing company then it becomes

apparent that QA/QC staff can become a large proportion of the workforce. Another practical aspect of GMP is validation. This simply means that all equipment and processes must be shown to perform as required. Thus, for example, all temperature control equipment such as refrigerators, freezers, incubators and autoclaves must have been monitored under load conditions and their performance documented. Any adjustments necessary to the controls to achieve the required performance must then be validated. The settings required, for example to run an incubator at 37 °C, should be displayed on the equipment along with a re-validation date. In addition, equipment crucial to the process such as incubators and freezers must be monitored constantly, with alarm limits, so that personnel can be warned, particularly outside working hours, if the equipment is out of specification. Further aspects of validation are discussed in Section 20.4.

20.2.3 MANUFACTURING CONDITIONS

The manufacture of sterile products must take place in a controlled environment that presents no hazard to the product. Risk assessment of the components of the process is undertaken to determine the standard of cleanliness required. As with everything else, there are guidelines to follow and standards to be met [1]. Cleanliness is measured as the number of particles of a range of sizes per cubic metre and there are several sets of classification depending on whether European or US Federal standards are being adhered to. Particle sizes of specific importance are $\geq 0.5 \mu\text{m}$ and $\geq 5.0 \mu\text{m}$. The requirements for cleanrooms include not only limits on particle count, but also the frequency of monitoring and the minimum pressure differential allowed between different areas (see Table 20.5). The European Guide [1] has three grades of Cleanroom: (i) A/B which corresponds to Class 100, M 3.5, ISO 5, (ii) grade C, which approximates to Class 10,000, M 5.5, ISO 7, and (iii) grade D, which is equivalent to Class 100,000, M 6.5, ISO 8.

Most gene therapy products cannot be terminally sterilised, therefore aseptic operations will be required throughout. Examples of such operations are aseptic preparation and filling, which would be carried out in a grade A environment, and the preparation of solutions to be filtered that could be handled in a grade C environment. Closed fermentation systems such as hard-piped bacterial or mammalian cell fermenters can be run in Class D rooms. Cell culture using disposable systems and small-scale fermentation are undertaken in Class C rooms, while open culture manipulations and final filling are performed under Class A/B conditions, which are often provided by a class II safety cabinet or isolator situated in a Class C room.

In addition to particle contamination, the bioburden in a cleanroom must be monitored regularly. It is generally true that cleanrooms can be run to very high standards as long as no one is working in them. The greatest

Table 20.5 Environmental limits and monitoring of cleanrooms

Grade	Maximum particles permitted/ m ³ (≥) (at rest)		Maximum particles permitted/ m ³ (≥) (occupied)		Sampling area (m ²)	Minimum pressure difference (Pa)		Monitoring frequency		
	5.0 μm		5.0 μm			a			b	
	0.5 μm	5.0 μm	0.5 μm	5.0 μm						
A	3 500	0	3 500	0	10	15	10	Weekly		
B	3 500	0	350 000	2 000	25	15	10	Weekly		
C	350 000	2 000	3 500 000	20 000	25	15	10	Monthly		
D	3 500 000	20 000	Not defined	Not defined	50	15	10	Quarterly		

a. between classified area and unclassified area.
b. between classified area and adjacent area of lower classification.

contaminant source is not usually the product but the personnel. Thus, there are strict guidelines for gowning, again according to the class of the room. For example, grade C gowning consists of a coverall gown with attached or separate hood, attached or separate leggings/foot covers, facemask and gloves, which ensures that minimal bare skin is exposed. Each facility has to build up a historical record of the bioburden in its cleanrooms on which to base its own alert and alarm limits. These limits will depend on the type of work carried out along with an assessment of the risk to the product. It is also necessary to determine the ability of the room to deal with a challenge by releasing particles and monitoring the length of time it takes to return to normal.

The cleanroom environment can be controlled in a number of ways. Firstly, the fabric of the room must not shed particles. Vinyl clad walling is usually employed, which provides a smooth, easily-cleaned surface that can be sealed where panels are joined together. Coving at the floor and ceiling ensures that there are no hard corners for dust to accumulate in. The air into the room is filtered to remove particulates and microorganisms (HEPA for grades A, B and C) and the room is maintained at a pressure positive to adjacent rooms (see Table 20.5). There must be a minimum of 20 air changes per hour, the actual number being related to the size of the room, equipment and personnel present. This can be assisted by the presence of a class II safety cabinet or isolator. Balancing air pressures and air flows is the cleanroom engineer's nightmare. Any equipment or process taking place in the room should be examined for its potential to create particulates or aerosols. All cleaning materials such as wipes must not shed particles and likewise any cleaning agents used should not leave residues, which could then be shed from the cleaned surfaces.

Correct cleaning is essential for maintenance of the cleanroom environment. There should be a disinfectant policy in place in which two or more agents are alternated and with documentation to record all cleaning operations. Where different products are manufactured on a campaign basis, fumigation and/or thorough cleaning between operations must be performed and validated, again with full documentation, and finally there should be release of the room for production use by the QA manager or equivalent.

Training in handling process equipment and in the principles and practice of working to GMP is necessary and must be documented, with a record for each employee, and there must be regular updates or re-training as required should a problem traceable to personnel behaviour arise. While training is an important aspect of working to GMP, it is vital when operating in cleanrooms. All procedures and behaviour must be geared towards protecting the

product from extraneous contamination. Thus, aseptic technique must be practised throughout where exposure of the product occurs, and particularly during the final fill of a sterile product. In gene therapy, the number of doses produced per batch is often too small to be handled mechanically, therefore manual filling of vials or manual control of bottle filling is usually practised. As with any sterile filling process, it is necessary to perform a number of media-only fills under normal operating conditions to validate that aseptic filling can be accomplished.

A further consideration when handling 'live' biologicals is containment. Under normal laboratory conditions, containment is achieved using negative pressure but in cleanrooms the maintenance of the environment depends on positive pressure. Containment within the room is therefore achieved using a class II safety cabinet or isolator while containment between rooms can be ensured by the use of a negative 'sink' area, usually the change area in which personnel gown up prior to entering the cleanroom environment. A further refinement is to construct the cleanroom walls with a negative pressure cavity, thus compensating for any leakage in the walls between adjacent cleanrooms.

Most viral vectors are 'disabled' in some way, usually with their replication genes deleted so that infectious virions are not assembled in normal cells. Thus, they are usually categorised for containment as Level 2.

20.3 MANUFACTURING STRATEGIES

As can be observed from the earlier chapters, many viruses are being investigated as the basic starting point for gene therapy vectors but they all require the same conditions for their production – the culture of (usually) mammalian cells. Hence, in principle, any one of these viruses could be produced using the same techniques. There are, however, a number of other factors to consider. Some viruses are cytopathic and kill infected cells; they may or may not remain associated with the cell debris. Other viruses are constitutive, e.g. retroviruses, and are secreted either throughout the life cycle of the cell or only during the growth phase of the cell, depending on the host cell origin. While some viruses are temperature sensitive and do not readily withstand freezing and thawing, others are more robust. Depending on their actual usage, e.g. *ex vivo* transduction or *in vivo* administration, there may or may not be a requirement for further purification. Thus, what seemed to be a fairly straightforward manufacturing process has quickly turned into a network of 'ifs' and 'thens'. Nevertheless, by the use of a limited number of cell culture options, it is possible to adapt the subsequent handling of the cultures to take account of most of these variables.

20.3.1 CELL CULTURE OPTIONS

Most of the cell lines in use for virus production are adherent; that is, they will stick to suitably treated surfaces, usually based on styrene. This allows the use of disposable plastics for cell growth, which has great benefits in reducing the amount of cleaning validation required. However, there are limitations in the amount of scale-up possible and these will be dealt with below. On the other hand, large-scale fermentation of mammalian cells, either in suspension or on microcarriers, has already been developed for vaccine and monoclonal antibody production and there is no reason why these procedures should not be adopted for growing some virus vectors once the demand for large quantities has developed.

20.3.1.1 Roller Bottles

Roller bottles have been the next size up from tissue culture flasks for many years and they are useful for production of relatively small quantities of virus or for multiple harvests of secreted virus. However, they are labour intensive, with the concomitant risks of contamination at each handling, although robotic handling systems can be installed if this is the method of choice. In addition, there is little control over the culture conditions within each bottle, and a change of conditions, e.g. to serum-free or selective medium, is not readily executed.

20.3.1.2 Costar Cellcube®

The Cellcube® consists of a multi-layered cube constructed of the same styrene as tissue culture flasks and connected to a circulation system that re-oxygenates the medium as well as controlling its pH. In size, the cubes can range from the equivalent surface area of 10 roller bottles up to 100 roller bottles. Even larger cubes are now available and they can be joined in parallel to a single circulation system. However, there is no temperature control mechanism and the apparatus must be sited within an incubator or warm room. Although the cube and tubing are disposable, the probes (pH and DO) and oxygenator are not, necessitating validated cleaning if the system is to be used for multiple products. However, a completely disposable system is under development.

20.3.1.3 Microcarriers

Several types of microcarrier are available. Some are porous, allowing cell growth throughout the structure, while others allow only surface growth.

This may be significant if the cells need to be grown then infected, e.g. for adenovirus production, since internal cells may not be accessible to the virus. However, where the vector is produced constitutively this is not a problem. There are many fermenter manufacturers producing vessels, ranging in scale from 1 litre to 10 000 litres, in which microcarriers can be grown. Culture conditions are fully controllable and there is industrial expertise already available using such fermenters for vaccine and biomolecule production.

20.3.1.4 New Brunswick Celligen ®

This is a fermenter system which uses as support medium for the cells a packed bed of polystyrene disks, contained within a basket in the vessel, which promotes the adherence of many cell types. Medium is forced through the basket, thus feeding and oxygenating the cells. The fermenter base has a water jacket, which heats the culture, therefore no incubator is required, while culture conditions are controlled and recorded by computer using New Brunswick software. This is most likely to be of use in producing secreted virus over long periods of time using a continuous feed system, with the product being stored in a refrigerator or led straight into downstream processing as required.

20.3.2 HOST CELL BANKS

Fully qualified banks of the host cell prepared under GMP conditions are a prerequisite for GMP production of virus. Testing for viral contaminants should be extensive to ensure contaminant-free production of the required vector. Both master and working cell banks will be required, and limits should be set on the number of passages allowed from culture initiation to viral infection as part of the control of the process [4].

For constitutive viruses, the host cell bank is also the virus bank. Again, limits should be set on the number of passages or harvests taken, and it is important to freeze down a post-production cell bank for each batch of virus so that tests for the emergence of replication-competent viruses can be performed.

20.3.3 VIRUS BANKS

Similarly, virus master and working banks should be laid down, titrated and tested for contaminants, including replication-competent viruses. Regular monitoring of the stored stocks is necessary, with replacement of the working stock as required.

20.3.4 VIRAL DOWNSTREAM PROCESSING

At present, relatively little processing is carried out. This has implications further upstream since there is little chance to remove extraneous organisms during the purification, as can happen with other biologicals such as recombinant proteins or blood products. Rigorous screening of all starting materials, especially those of biological origin, including the host cell and the seed virus, is therefore essential [5].

If the virus is released or secreted into the medium then facilities for handling large volumes of infectious material will be required. In this case centrifugation is not an option unless continuous flow methods are used, but cell debris can be removed using suitably sized cross-flow membranes followed by virus concentration using a size-exclusion membrane. These procedures are relatively gentle and can be performed at 4 °C to increase virus stability. They are also applicable to large volumes, and process times are not so long as to affect virus stability.

For viruses which remain cell or nucleus associated, some means of their release must be sought. At the laboratory scale this is usually performed by freeze–thaw or sonication. However, there may be problems in scaling up these methods – large volumes may shield the inner cells from freezing and thawing sufficiently rapidly to cause cell rupture, while sonication can create ‘hotspots’ leading to virus degradation unless adequate cooling with stirring is applied.

Some viruses form a large proportion of ‘empty’ virus particles, which must be removed before their administration to patients. This is especially the case with adenovirus in which the outer antigen is inflammatory, and there are regulatory requirements to minimise the particle to infectivity ratio to less than 100:1. The classical method of adenovirus purification has been separation by density gradient centrifugation using caesium chloride, a toxic substance which must then be removed from the preparation by dialysis or desalting chromatography. The scale-up possibilities of this method are obviously limited, and a number of chromatographic-based methods are currently under investigation. As greater experience in administering adenovirus for gene therapy is gained, so it is becoming apparent that the quantities required may be of the order of logs of magnitude greater than earlier studies had indicated would be tolerated. Thus, improvements in the scale-up of adenovirus production and purification are fast becoming a priority.

20.3.5 VIRUS STORAGE

Most virus preparations are stable when stored frozen at temperatures below –75 °C. More convenient, however, would be storage at 4 °C but only limited

studies have been undertaken on the long-term effects of this temperature on viral vector infectivity. Undoubtedly, stabilisation additives would be necessary. Freeze drying is an attractive option, again if the appropriate additives can be identified.

20.3.6 BACTERIAL FERMENTATION

The source of DNA for the majority of gene therapies takes the form of plasmid DNA, whether in its native supercoiled form, linearised or incorporated into particles such as liposomes. Plasmids are usually propagated in engineered forms of *E. coli*. Bacterial fermentation has been a manufacturing process for numerous years for many different products. However, growth of the bacterium must be carefully controlled so that neither non-plasmid-bearing forms grow out nor are the plasmid sequences corrupted. Thus, a fine balance between bacterial biomass and the number of plasmid copies has to be struck.

As for cell culture, there are many manufacturers of bacterial fermenters producing vessels ranging in scale from one to many thousands of litres in size. Oxygenation and pH of the process can be closely controlled using computer software, while other parameters such as biomass or glucose concentration will be controllable as more sophisticated monitoring systems are developed.

20.3.7 PLASMID DOWNSTREAM PROCESSING

Suitable equipment must be installed that can handle large volumes of bacterial culture with containment until that part of the process in which the cells are killed is reached. Some form of cell harvest or concentration is necessary, and equipment such as continuous-flow centrifuges or spin filters can be used. Once the concentrated cell pellet has been lysed the material can then be moved to another area for further processing, but the cell killing must have been validated. Cleaning of the downstream area and its validation is then facilitated if the process can be confined to a closed system using hard piping and closed containers. Thus, only the fermentation and harvest equipment will require decontamination as well as cleaning.

Current plasmid purification consists of alkaline lysis of the bacteria followed by a series of precipitation steps and one or more chromatographic steps. As with any pharmaceutical process, each step must be controlled and monitored by appropriate methods with specifications set which must be met before the batch is cleared for further processing. This requires close cooperation between production and quality control staff so that unnecessary delays are not encountered.

20.4 EQUIPMENT

According to the GMP guidelines [1], all equipment must be designed, located and maintained to suit the intended purpose. At no time must it constitute a hazard to the quality of the product and it should be designed to allow thorough cleaning, which must be carried out according to written procedures. New equipment or equipment moved from another site must go through a series of qualifications before its use in the manufacturing process. These are known as Installation Qualification (IQ), Operational Qualification (OQ) and Performance Qualification (PQ), and are based on the validation sub-categories suggested by the US FDA 'Guidelines on General Principles of Process Validation' published in 1987 [7]. Each stage consists of documentation that shows that for the equipment or facility being validated the installation conforms to the design specification (IQ), operates correctly within specified limits (OQ) and performs reliably and consistently or produces a product that meets predetermined specifications (PQ). These qualification and validation exercises can be used to prepare an instruction manual as well as providing personnel training and experience in the operation of the equipment.

For large pieces of equipment or new premises, formal acceptance will not take place until at least the IQ and OQ have been completed satisfactorily. Problems in completing the PQ may arise if the original design specifications were inappropriate, and modification of the design or process may therefore be necessary.

Once it has passed, the equipment must be monitored regularly to ensure that the fixed processing parameters are being maintained. This may involve a variety of instrumentation types, which must themselves be calibrated accurately if the monitoring data is to be accurate. Following on from this is the necessity for a regular preventive maintenance programme.

20.4.1 MASTER VALIDATION PLAN

A validation project, especially for a new building or process, can be a lengthy and costly exercise. It therefore makes sense to gain as much as possible from it, and this can only be achieved by good preparation beforehand. A Steering Committee should be appointed consisting of a small group of highly experienced personnel whose remit will be to prepare the Master Validation Plan and approve the protocols. For a specific process or project, a Working Group will then prepare the justification for validation, write the protocols, estimate costs and carry out the validation studies. Written records should be maintained of all that is discussed, including what it is decided *not* to do as well as what to do.

A properly prepared and executed Master Validation Plan provides many benefits:

- better understanding of systems/processes
- a structure for validation activities
- coordination of activities
- information for senior management, regulators, contractors etc.
- a foundation for process control and monitoring
- aid in protocol preparation
- allows corrective action to be taken
- identifies resource/staffing issues
- assists with audit preparation
- encourages good communication.

Table 20.6 illustrates some of the topics to be covered by a Master Validation Plan document.

20.4.2 CLEANING

Validation of equipment cleaning is critical where equipment is used for multiple products. Where possible, in such a situation, use should be made of sterile plastic disposables, such as liners for containers or closed systems such as bags. Where multiple usage is unavoidable, a mixture of product-specific and non-specific detection of contaminants may be employed [8]. Total organic carbon (TOC) analysis provides rapid results and is extremely sensitive [9].

20.5 DOCUMENTATION

A documentation system must be set up that allows full traceability of the entire process, from starting materials to finished product and all stages in between. It can be conveniently broken down into sections and these are illustrated in Figure 20.1. Standard operating procedures (SOPs) provide the backbone of principles on which the rest is based [1]. These set out the standards to be achieved, how procedures and documentation will be controlled, monitoring of equipment and the environment, equipment preparation etc. From this, specific protocols can be prepared to cover individual aspects of the process, e.g. cleaning of a particular piece of equipment or the preparation of a buffer.

A standard format should be adopted throughout, with the title, nature and purpose of the document clearly stated, and it should be approved,

Table 20.6 Suggested contents for master validation plan

1.0	Introduction and scope	Document approval
2.0	Glossary of terms	
3.0	Facility description and design	
3.1	Location and general	
3.2	Design concept description	
3.3	Materials of construction	
3.4	Room classifications	
4.0	Process description	
4.1	Production process	
4.2	Materials flow	
4.3	Personnel flows	
4.4	Process control	
5.0	Services and utilities	
5.1	General utilities	
5.2	Specialist water systems	
5.3	Autoclaves	
6.0	Equipment and systems to be validated	
6.1	Validation concept	
6.2	Design validation	
6.3	Equipment and systems validation matrix	
7.0	Acceptance criteria	
7.1	General acceptance criteria	
7.2	Facility	
7.3	Utilities and services	
7.4	Process equipment and systems	
8.0	Required procedures	
8.1	List of SOPs required	
8.2	List of validation protocols required	
8.3	Training programme	
8.4	Instrument calibration	
8.5	Planned maintenance	
8.6	Change control	
9.0	Protocol and documentation format	
9.1	General requirements	
9.2	Validation protocols	
9.3	Deficiency reports	
9.4	Validation reports	
9.5	Validation project file	
9.6	Examples of validation documents	
10.0	Management and resources	
10.1	Validation management	
10.2	Outline validation programme	
10.3	Validation resources	
11.0	Equipment and system master files	
11.1	General requirements	
11.2	Master File contents sheets	
12.0	Project sign off	
13.0	Drawings	

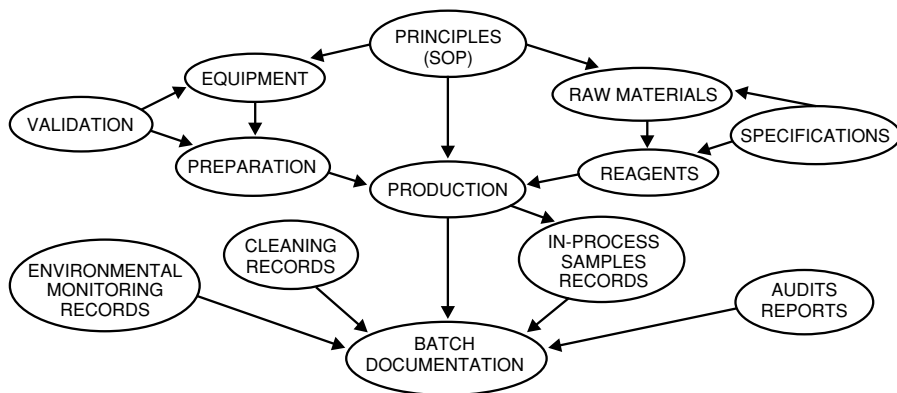


Figure 20.1. Documentation network.

signed and dated by the appropriate personnel. A revision system should be in place and control kept of changes such that outdated documents cannot be issued inadvertently. Staff should be trained in the proper use and completion of documents, and they should complete each section at the time of execution, noting any changes or deviations in the procedure with a signature and date, and an explanation where required. QA staff will then audit the protocols and give their formal approval. Audits of procedures will also be undertaken by QA and written reports prepared and approved. These then become part of the batch documentation. Archiving of documentation is essential and there must be written procedures detailing storage and retrieval.

Electronic recording of data is allowed but there must be appropriate safeguards to ensure the integrity of the data and there must be no decrease in either product quality or quality assurance. In addition, computer systems require to be validated as equipment. Data must be protected by regular back-up and checked for accessibility, durability and accuracy. Alternative arrangements must be available to take over in the event of a breakdown, and these procedures should be defined and validated.

20.6 BUILDING DESIGN

Design and construction of a suitable building to house these activities is a major undertaking. Cost is obviously a prime consideration and contract manufacturing may be a sensible option for early clinical trials material. However, should there be a requirement for a production facility, then flexibility will be the key to success, bearing in mind the current uncertainty

of the final format of gene therapy products. Whether the facility is custom-built or redeveloped using existing buildings is the first decision.

Provision of services such as Water for Injection can be prohibitive for a small company and the use of packaged sterile water from an approved source may be a more cost-effective alternative.

Similarly, the provision of a pure steam supply for autoclaves or fermenters may be expensive if there is no plant steam available in the building. Stand-alone steam generators may be a cost-effective option where requirements are minimal.

Cleanroom grades will be based upon the process envisaged, as outlined earlier, and linked to this will be the air handling system. Airflows and pressure differentials must be carefully designed to ensure that containment cannot be breached when materials or personnel move from one room to another. There should be continuous monitoring and control of the rooms by a building management system (BMS) with alarm limits set to detect and warn of changes in conditions. Likewise, there should be temperature and other appropriate monitoring of process and storage equipment.

Space should be allocated for storage of raw materials and prepared reagents, materials in quarantine, passed or rejected, samples, archived material and product, in addition to equipment not currently in use.

Waste management, particularly of potentially infectious material, is an important aspect and there should be a clearly defined flow of waste to ensure that product cannot be contaminated by contact. Decontamination of liquid and solid waste must be validated, and disposal should follow local regulations.

20.7 SUMMARY

This chapter has attempted to discuss many of the issues involved in providing facilities for production of gene therapy products. While the basic cell culture and fermentation methods are available now, there remain major problems in increasing virus yield, in viral downstream processing and in long-term viral stability. In particular, methods suitable for scale-up to full manufacturing proportions, as opposed to clinical trials, need to be developed. Flexibility will undoubtedly be the key to success for current and future manufacturing facilities since at present the final format(s) of gene therapy products remains unclear.

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Addendum

Since the present authors were recruited to contribute chapters for this book a number of papers with relevance to quality assurance in gene therapy have appeared in a special issue of the *Quality Assurance Journal* (2(3), September 1997) © John Wiley & Sons, Ltd. They are:

Sajjidi, N. Development of retroviral-based gene therapy products: a quality control perspective. *Qual. Assur. J.* 2: 113–117, 1997.

Hutchins, B. Making the (clinical) grade with adenoviral gene therapy vectors. *Qual. Assur. J.* 2: 119–127, 1997.

Kuhne, W. and Zollner, K. Recommendations for the successful quality assurance of production of bulk drug substances for non-viral gene therapy. *Qual. Assur. J.* 2: 129–133, 1997.

Seitz Webster, H. Compliance issues for the contract manufacturing of cGMP gene therapy products. *Qual. Assur. J.* 2: 135–140, 1997.

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